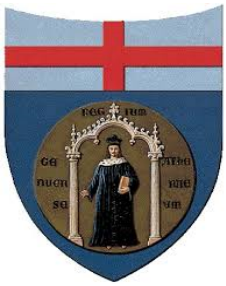

UNIVERSITÀ DEGLI STUDI DI GENOVA

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CENTRE OF EXCELLENCE FOR BIOMEDICAL RESEARCH

XXXII Cycle

PhD in Clinical and Experimental Immunology

EXPERIMENTAL THESIS

Effect of Tyrosine kinase inhibitors on NK cell and ILC3 development and function

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INDEX

1.INTRODUCTION

1.1 Natural Killer cells

Natural Killer cells (NK cells) are the main subset of Innate Lymphoid Cells (ILC) and represent the 5-15% of circulating lymphocytes in peripheral blood. NK cells show a high potential in tumor surveillance and in defense against viruses (e.g. herpes virus) [1-3]. Tissue-resident NK cells have been found in liver, uterus, and decidua. NK cells have the ability to migrate to secondary lymphoid organs (SLOs) and to inflamed tissues where they contribute to the first line of defense against various pathogens [2, 4-6]. After activation, NK cells release chemokines such as MIP-1α/β, CCL5, CXCL1 and cytokines such as IFN-γ, TNF- α, GM-CSF, IL-10 which can mediate/increase not only inflammatory responses but also contribute to hematopoiesis and activation of granulocytes and monocytes. Moreover, NK cells can also interact with dendritic cells controlling their functional maturation, thus influencing the adaptive response of Th1 lymphocytes [2, 5].

Peripheral NK cells are composed of two major subpopulations that can be identified through the level of surface expression of CD56 molecule and on the basis of their receptor repertoire, functional characteristics and tissue localization. The $CD56^{dim}$ cells is the most differentiated and cytotoxic subset that shows a high expression of CD16 and KIR inhibitory receptors. They produce lower levels of cytokines as compared to $CD56^{bright}$ and reside in the peripheral blood. The $CD56^{bright}$ cells express CD94/NKG2A inhibitory receptor well but not KIR, they display low or any expression of CD16 and produce high levels of cytokines. They are found mainly in SLO (e.g. lymph nodes). They may represent precursors of $CD16^{dim}$ NK cells [7, 8].

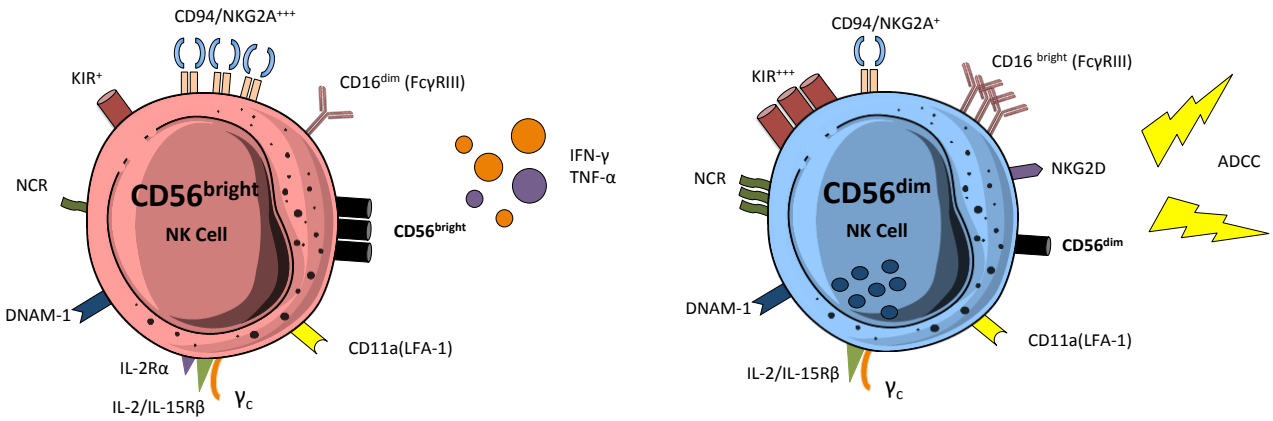

Figure 1. NK cells subsets: $CD56^{dim}$ cytotoxic NK subset express low levels of CD56 molecule and are able to perform ADCC thanks to the high expression of CD16 receptor;

CD56^{bright} NK subset express high levels of CD56 molecule and is capable to produce IFN-γ and TNF-α, thus contributing to the activation of monocytes and Th1 response.

1.2 Activating receptors/co-receptors and cytolytic activity

NK cells exert cytotoxic activity and cytokines release through a balance between activating and inhibitory receptors [9-12].

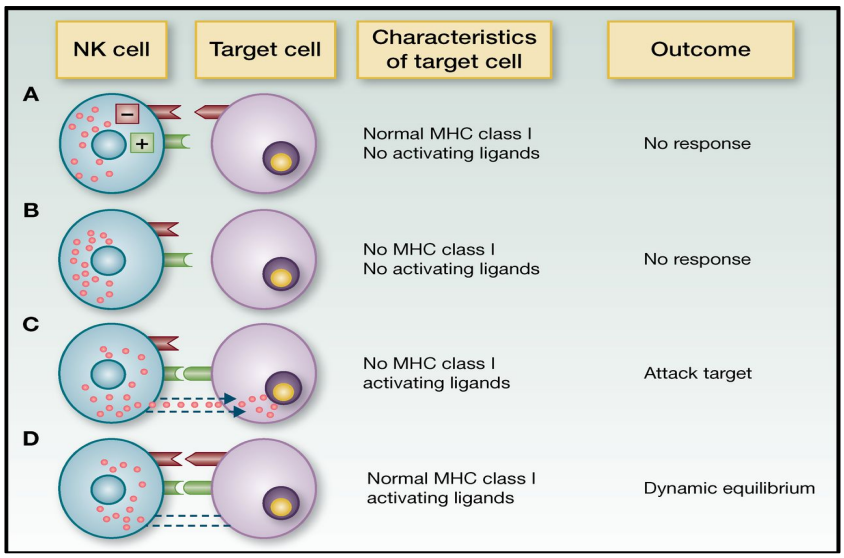

Figure 2. Cytotoxic activity of NK cell against tumor cell: A) the target cell cannot express ligands for activating receptor and express normal levels of Human Leukocyte Antigen class I molecules (HLA-I) blocking NK cell response; B) In the absence of HLA class I molecules NK cell cytotoxicity can be inhibited by the lack of expression of activating receptor ligands on tumor cells. C) NK cell activity is exerted by the binding of ligand to activating receptor and thanks to the lack of HLA-I molecules on target cell; D) the normal expression of both ligands leads to a dynamic balance between NK cell and target cell. (Adapted from Leung Wing, 2014)

NK cells can perform their lytic functions through ADCC activation or through the activity of Natural Cytotoxicity Receptors (NCR). The recognition by CD16 receptor of IgG-opsonized cells activates the signal transduction cascade that involves molecules such as CD3ζ, FcεRIγ, SyK and ZAP70. This signal cascade terminates with ERK1/2 phosphorylation that promotes polarization of lytic granules containing Perforin and Granzyme A/B and then their exocytosis [13].

NCR were identified on NK cells in 1990s. These receptors have the ability to recognize specific ligands on virus-infected cells or tumors [14, 15]. These Ig-like receptors includes NKp30, NKp44 and NKp46; only some ligands of these receptors have been identified including BAT3 and B7H6 (NKp30-L), Mixed-Lineage Leukemia-5 MLL5, Sindecan-4, Galectin-3 and soluble ligand NID1 (NKp44-L) and CFP and other viral ligands (NKP46-L). The adapter proteins such as FcεRIγ, CD3ζ and DAP12, specifically related to NCR, containing immunoreceptor tyrosin-based activation motifs (ITAM), are responsible for the activation signal. NKp44 displays some peculiarities: its cytoplasmic tail contains immunoreceptor tyrosine-based inhibition motifs (ITIM) that is capable to inhibit the release of cytotoxic agents and IFN-γ [16-18].

DNAM-1 and NKG2D are other important activating receptors that recognize ligands, PVR (CD112), Nectin-2 (CD155) and MICA/B, ULBPs respectively, that are hyper- or *de novo*-expressed only by tumor/stressed cells [10, 19].

NK cells express also activating co-receptors on their surface such as 2B4 (CD244), NTB-A (CD352) and NKp80 [20-22]. The 2B4 receptor belongs to the family of signaling lymphocyte activation molecule (SLAM)-related receptors (SRR) and recognizes CD48 molecule. This receptor shows a long cytoplasmic tail containing tyrosine residues that if phosphorylated allow the association of SLAM-associated protein (SAP) and therefore the activating signal transduction. The lack of SAP expression triggers an inhibitory signal with consequent reduction of cytotoxic activity and production of IFN-γ (condition that characterized X-linked lymphoproliferative syndrome, XLP) [21, 23]. NTB-A protein is similar to 2B4 protein both structurally and functionally, but the activation requires a homophile bond with another NTB-A molecule [22].

Finally, NKp80 receptor, expressed as dimer, recognizes AICL molecule (expressed by activating monocytes and tumor cells) and presents a lectin domain in extracellular portion and tyrosine-based motifs in the intracellular portion. The interaction with ligand leads to activation of NK cytolytic activity [20].

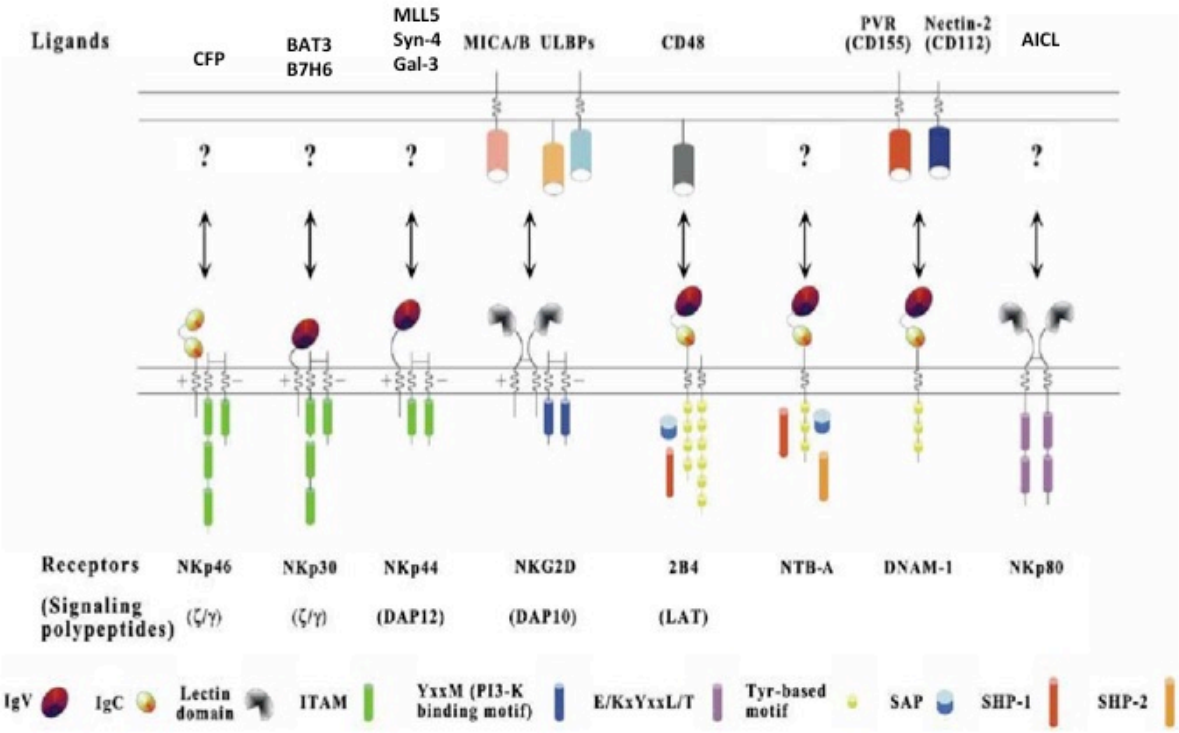

Figure 3. Activating receptors, co-receptor and their ligands: NKp46, NKp30 and NKp44 (NCR), containing ITAM in the intra-cytoplasmic portion, are associated with ζ/γ signaling protein, which is responsible to activating signal. NKG2D is a C-type lectin receptor that presents YxxM intra-cytoplasmic PI3K-binding motifs and is associated to DAP10 adaptor protein for signal transduction. DNAM-1, 2B4 and NTB-A have IgV and IgC extracellular domains. NTB-A and 2B4 translate the activating signal thank to the involvement of SAP and SHP-1 proteins. NKp80 have two C-type lectin extracellular domains and E/KxYxxL/T motifs in intracellular chains. (Adapted from Moretta A et al, 2004)

1.3 Inhibitory receptors and their HLA-I ligands

The inhibitory counterpart of NK cell receptors is represented by specific molecules including killer Ig-like receptors (KIR), C-type lectin receptor CD94/NKG2a and Leukocyte immunoglobulin-like receptor subfamily B member 1 (LILRB1 or LIR1) [14, 24, 25]. The receptor CD94/NKG2a recognizes the expression of HLA-E (non-classical HLA-I molecule) on cells by binding peptides derived from leader sequence of classical HLA-I molecules [11, 14, 24, 26]. The gene of NKG2a is located on chromosome 12.

It exists an activating counterpart, named CD94/NKG2c that recognizes the same molecules and it is associated with ITAM motifs and DAP12 adaptive protein for activating signal transduction. It has been observed that HCMV infections promote the oligoclonal expansion of mature NK cells expressing high levels of NKG2c receptor [27, 28].

LIR1 inhibitory receptor can contain 2 or 4 Ig domains and 2 to 4 ITIM; its gene resides on chromosome 19q13.4. This receptor binds HLA-I molecules and non-classical HLA-I molecules leading to an inhibitory signal [24].

KIR receptors are clonally distributed, their genes (family of 13 genes) are located in Leukocytes Receptor Complex (LRC) on chromosome 19q13.4 and are inherited as haplotypes. There are two KIR haplotypes: haplotype A is characterized by KIR2DL1, KIR2DL3, KIR3DL1, KIR3DL2, KIR2DL4 genes encoding inhibitory isoforms, KIR2DS4 encoding activating isoform and two pseudo-genes KIR2DP1 and KIR3DP; haplotype B include KIR2DL2, KIR2DL5A, KIR2DL5B, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS5 and KIR3DS1 genes. Inhibitory KIR (iKIR) are identified by 2 (KIR2D) or 3 (KIR3D) Ig-like domains in their extracellular portion and by a short or long intra-cytoplasmic chain. The long cytoplasmic chains (KIR2DL and KIR3DL) are characteristic of the inhibitory isoforms and contain ITIM, which recruit protein tyrosine phosphatases that mediate inhibitory function [25, 29]. The activating isoforms (aKIR) are characterized by short cytoplasmic chains (KIR2DS and KIR3DS) containing ITAM associated to signaling adaptor protein KARAP/DAP12, which are implicated in activating signal transduction. It was observed that KIR2DS4 activating receptor is present only in some individuals and may also positively contribute to the anti-leukemia activity [29, 30]. Recently, Dumas PY et al suggested that KIR2DL5B genotype of CML patients has a role in the control of minimal residual disease (MRD) in patients with CML relapse [31].

HLA-I ligands are represented by HLA-A, HLA-B and HLA-C and their genes reside on chromosome 6 (except β2-microglobulin located on chromosome 15) [29]. The acquisition of KIR inhibitory receptors from NK cells undergoing differentiation from CD34 HSC after hematopoietic Stem Cell Transplantation (HSCT) may have an important role against

leukemia stem cells (LSC), also called minimal residual disease (MRD) [29, 32]. After 4 weeks, donor NK cells are completely functional and can express specific KIR receptors for donor HLA-I molecules and not recipient (mismatch) [29, 33]. This NK cells are defined "alloreactive NK cells" and have high lysis efficiency against any residual leukemic blasts [34-36]. The NK cells alloreactivity was first described more than 20 years ago by Moretta A et al [29, 32, 37].

During NK cells development, after interaction between KIR receptors and HLA-I "self" ligands, NK cells become educated/licensed to exercise alloreactivity against allogeneic targets that do not express HLA-I "self" ligands [29].

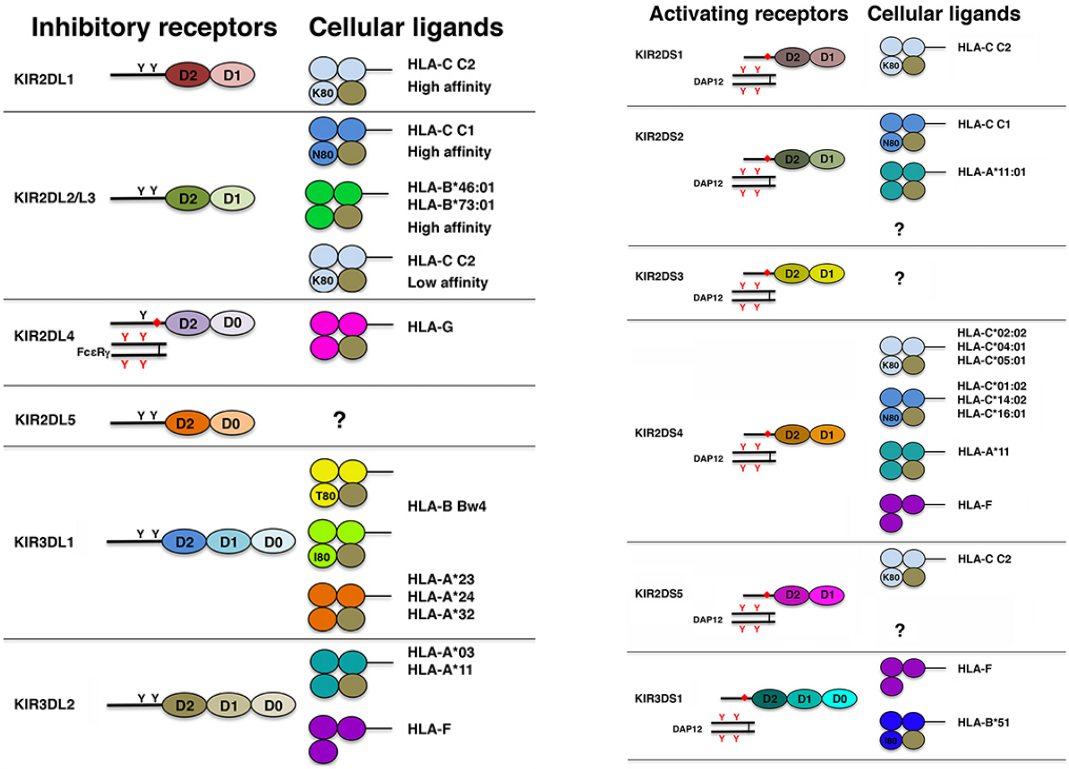

Figure 4. Killer Ig-like inhibitory and activating receptors consist of Ig domains: KIR2D encodes 2 domains with D1 and D2 conformation or D0 and D2 conformation; KIR3D encodes 3 domains with D0, D1 and D2 conformation. KIR2DL1, KIR2DL2/L3, KIR2DL5, KIR3DL1 and KIR3DL3 contain 2 ITIMs in intra-cytoplasmic tail. Differently from other iKIR, in addition to the presence of single ITIM, KIR2DL4 contains a charged residue in its trans-membrane region and 4 ITAM in associated protein FcεRγ, suggesting a possible activating role. KIR2DL4 is the only isoform that bind non-classical HLA-G molecule. (Adapted from Pende D et al, 2019).

1.4 Origin and development of ILC/NK cell

NK cell development occurs primarily in the bone marrow (BM) and in other sites such as thymus and lymph nodes and requires the expression of NFIL3, Tbx21, RUNX1, BLIMP1, EOMES, ETS-1, GATA3, TOX1 and ID2 transcription factors (TF) [8, 38-40]. This process is characterized by the sequential acquisition of surface markers (including CD161, CD56, CD94/NKG2a, LFA-1, CD16, KIRs and CD57) and functional capabilities [39, 41, 42]. Another important NK maturation marker is NKp80 that discriminates between stage 4a represented by IL-22 producing RORγt^{+}AHR^{+}ILC3 and stage 4b characterized by IFN-γ producing Tbet^{+}EOMES^{+} NK cells during development in secondary lymphoid tissues (SLTs) [20].

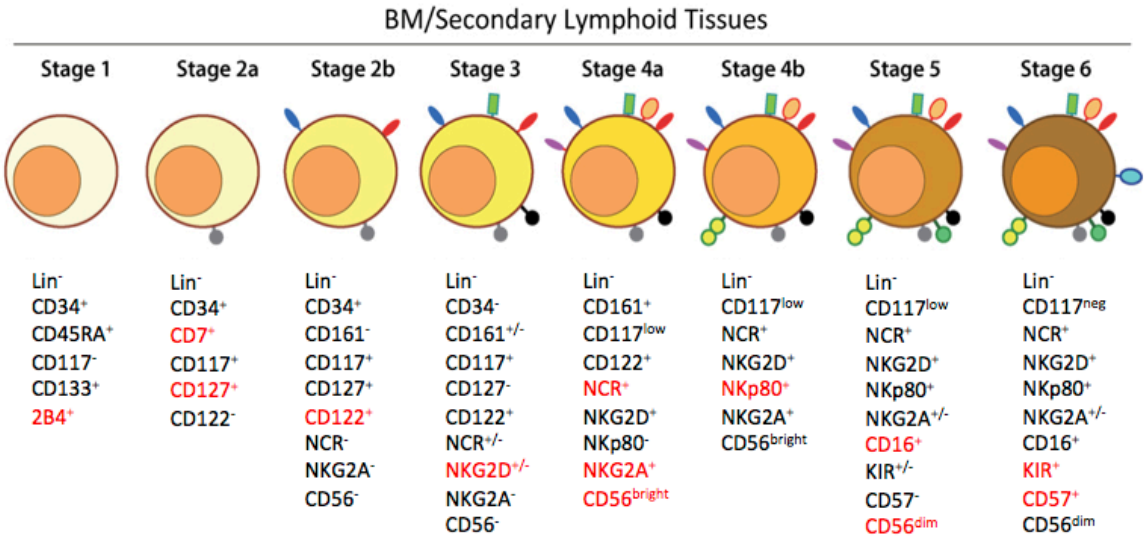

Figure 5: Six-stages NK cell development model: the specific markers that identify different steps are highlighted in red. Stage 1 and stage 2 characterized by CD34^{+} multi-potent cells that can give rise to dendritic cells (DC), T cells and NK cells. In particular, Stage 2b is an important step represented by IL-15 receptor (IL-15R) β chain (CD122) and IL-7 receptor (IL-7R) CD127 expression on NK cells precursors. The interaction between IL-15R and IL-7R and their ligands is essential for differentiation of cytolytic NK cells and homeostasis respectively. Stage 4 is divided into stage 4a and stage 4b depending on NKp80 surface expression and TF. Stage 3 and Stage 4a are represented by CD161$^{+/-}$ CD56^{-}CD117^{+} NK cells precursors and NCR expressing ILC3. (Adapted from Abel AM et al 2018)

NK cells have been recently included in a larger family of innate lymphocytes, called Innate Lymphoid cells (ILC) [43]. All of them derive from a common DNA-binding protein inhibitor (ID2)-positive hematopoietic precursor and both express IL-2Rγ (common receptor for IL-2, IL-7, IL-15 and IL-21) [39, 40, 44]. The ILC classification and nomenclature was defined in 2013 by Spits et al. ILC are divided into three groups according to phenotypic and functions properties [45]. NK cells are the only cytotoxic subset while other ILCs are characterized by the ability to produce several different cytokines. With the exception of NK cells, ILCs display a preferential tissue distribution: they are mainly found in gut, lungs, decidua and skin where they have a protection role in skin inflammations. ILC1 group is characterized by t-bet TF expression, production of Th1-associated cytokine IFN-γ; it resides in gut and has a defense role against intracellular pathogens and protozoa [45]. ILC2 group produces Th2-related cytokines such as IL-5 and IL-13 and for its development depends on GATA3, RORα and BCL11B TF; is involved in defense against parasites and in allergic disease. Finally, ILC3 group requires AHR and RORγt TF, may express NKp44 receptor and are activated by IL-23 and IL-1β to produce IL-17 and IL-22 Th17-related cytokines; primarily defined lymphoid tissue inducer (LTi) cells, seem to be involved in lymph node organogenesis during fetal life and in maintaining the integrity of intestinal epithelium during adult life, where they contribute to host defense against extracellular pathogens [43, 45-49]. In recent years, it has been observed a positive role of ILC3 during intestinal inflammation. However, ILC3 may also increase intestinal inflammation converting them into IFN-γ-producing ILC1 upon IL-12 stimulation [50, 51]. The constantly activation of ILC3 and thus, the high and sustained production of IL-17 and IL-22, have been suggested to promote colorectal tumor growth and metastasis [52, 53].

Several mouse and human *in vitro* and *in vivo* studies have shown that ILCs can convert in other subsets in the presence of specific cytokines [54]. In particular, the plasticity of murine RORγt$^+$ILC3 is well been demonstrated *in vivo*; murine ILC3s are divided in two majors' subsets, LTi cells and NCR$^+$ILC3 (and their NCR$^-$ILC3 precursors). NCR$^+$ILC3 are characterized by NKp46 surface expression, lack of CCR6 chemokines receptor expression and IL-22 secretion. LTi cells instead are specialized in IL-17 production. CCR6$^-$ NKp46$^+$ILC3 can convert to ILC1 responding to IL-12 (ex ILC3) and produce IFN-γ when stimulated with IL-2 or IL-15 [54, 55]. A study of Bernink JH et al shows that the reversion of ILC1 to ILC3 can be supported by IL-23 but the molecular process of this conversion remains unknown. Recent studies revealed that human ILC3, isolated from pediatric tonsils and from humanized mouse tissues could differentiate in NK cells stage 4 [51].

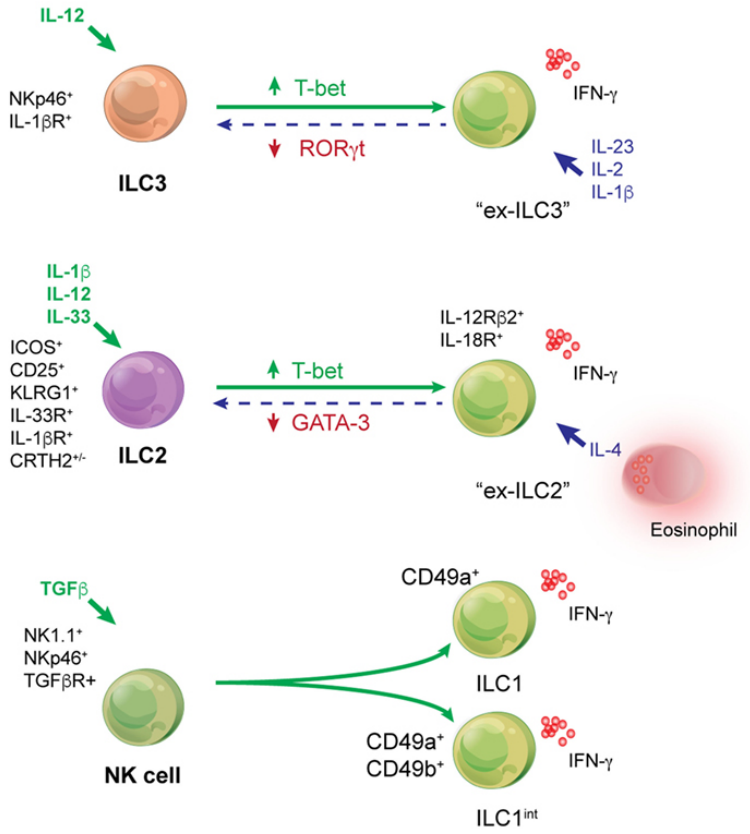

Figure 6. Plasticity of ILCs: IL-12 stimulates the phenotype and functional conversion of ILC3 to ILC1-like ("ex-ILC3") while IL-23 and IL-1β reverse to RORγt^{+}-ILC3. ILC2 can be converted into "ex-ILC2" thanks to IL-12 and IL-33 stimulations. IL-4 stimulates the conversion of "ex-ILC2" into GATA3^{+}-ILC2. NK cells respond to TGF-β acquiring CD49a and/or CD49b molecules to become ILC1 and intra-epithelial ILC1 (ILC1^{int}). (From Huang Q et al 2017)

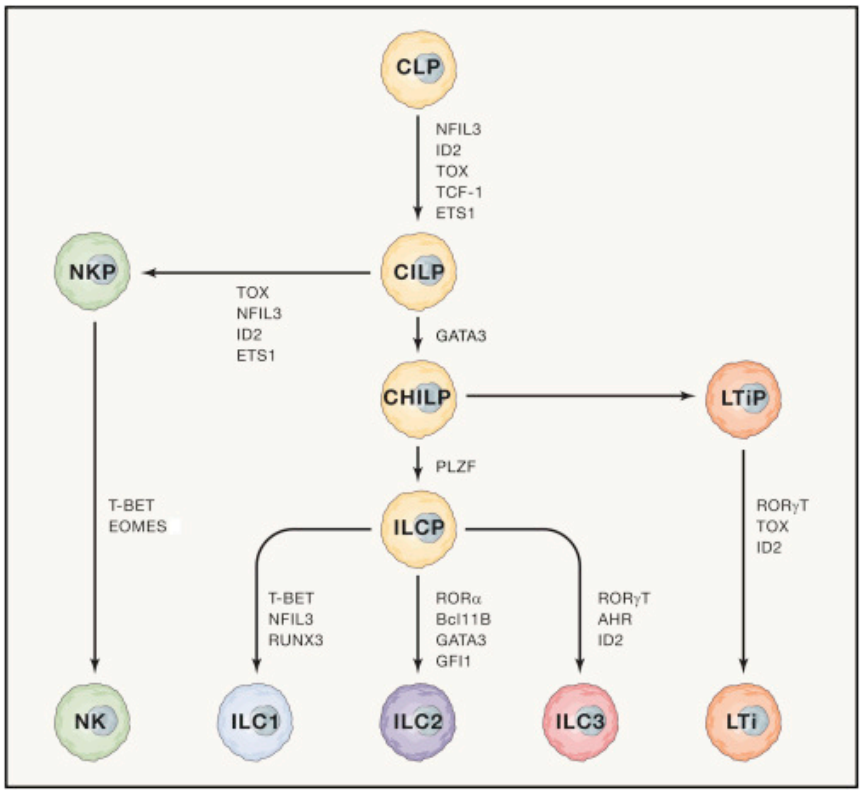

Figure 7. ILC and NK cells derived from Common Lymphoid Precursor (CLP) that differentiates in Common Innate Lymphoid Progenitor (CILP). CILP can give rise both to ILC/LTi precursor CHILP and to the NK cell precursor (NKP) through the specific TF. (From Vivier E et al 2018)

Stimuli		Mediators	Immune function
Tumors, intracellular microbes (Virus, bacteria, parasites)	→ NK ILC1	IFN-γ Granzymes Perforin	Type 1 immunity (Macrophage activation, cytotoxicity)
Large extracellular parasites and allergens	→ ILC2	IL-4 IL-5 IL-13 IL-9 AREG	Type 2 immunity (Alternative macrophage activation)
Mesenchymal organizer cells (Retinoic acid, CXCL13, RANK-L)	→ LTi	RANK Lymphotoxin TNF IL-17 IL-22	Formation of secondary lymphoid structures
Extracellular microbes (Bacteria, fungi)	→ ILC3	IL-22 IL-17 GM-CSF Lymphotoxin	Type 3 immunity (Phagocytosis, antimicrobial peptides)

Figure 8. ILC and NK cells immune functions: different stimuli activate NK cells and ILCs which through the release of cytokines and/or lytic granules that can mediate immune response. (From Vivier E et al 2018)

1.5 *In vitro* model of ILC3/NK differentiation

ILC3/NK cells develop from umbilical cord blood (UCB)-derived $CD34^+$ cells thanks to an appropriate stimulation that involves some cytokines such as stem cell factor (SCF), FMS-like tyrosine kinase 3 Ligand (FLT3-L), IL-7, IL-15 and IL-21[39]. This *in vitro* model can also give rise to $CD33^+CD14^{+/-}$ myeloid-monocytes cells and provides for five differentiation stages based on phenotypic and functional characteristics: stage I $CD34^+CD117^-CD94/NKG2a^-CD56^-$, Stage II $CD34^+CD117^+CD161^{+/-}CD94/NKG2a^-CD56^-$, Stage III $CD34^-CD117^+CD161^+LFA\text{-}1^-CD94/NKG2a^-CD56^+$ (immature NK cells), Stage IV $CD34^-CD117^-LFA\text{-}1^+CD94/NKG2a^+CD56^{bright}CD16^{dim}$ able to produce IFN-γ and Stage V $CD34^-CD117^-CD94/NKG2a^{+/-}CD56^{dim}CD16^{bright}KIR^{+/-}$ $EOMES^+$ which instead performs cytotoxic activity through release of cytolytic granules containing perforin and granzyme A/B. From the NK stage III can derive both $CD34^-CD117^+$ $LFA\text{-}1^-$ $CD94/NKG2a^-CD56^+$RORγt-ILC3 and NK stage IV [47, 56]. Lymphocyte function-associated antigen 1 (LFA-1 or CD11a) is an adhesion molecule belonging to the integrin superfamily. The interaction between LFA-1 molecule and their ligands such as intercellular adhesion molecule 1 (ICAM-1) and ICAM-2 leads to immunological synapse formation which is the fundamental requirement for NK cells to exercise cytotoxic activity [57].

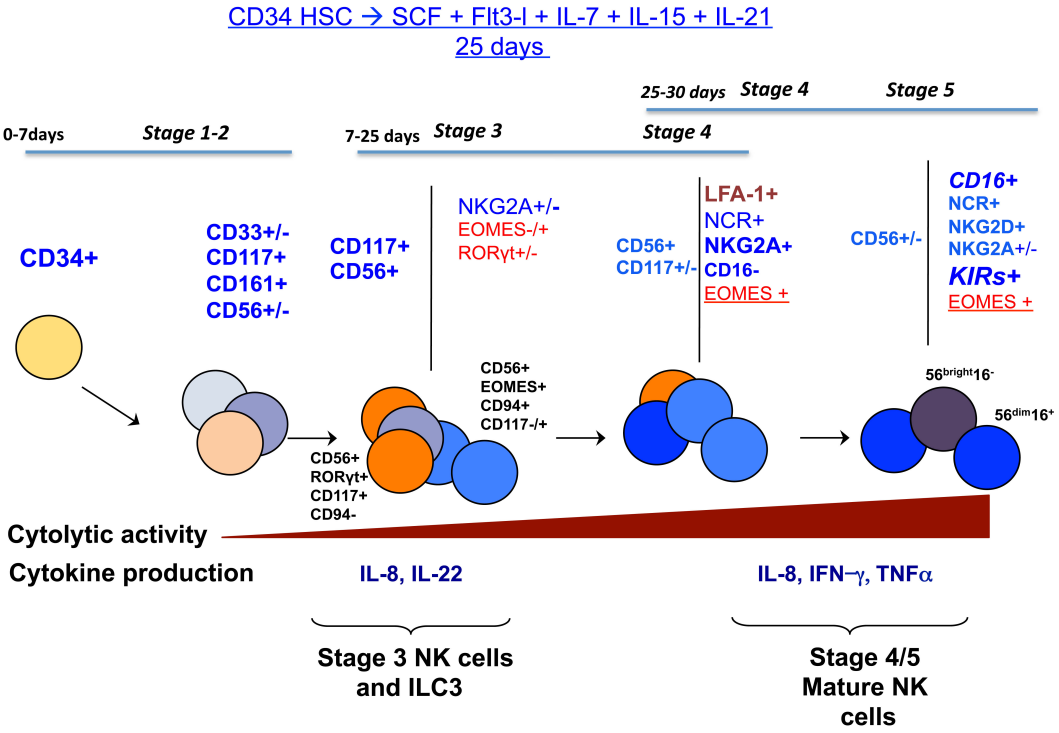

Figure 9. Five steps represent NK/ILC3 in vitro differentiation model. $CD34^+$ precursors isolated by UCB are cultured with SCF, FLT3-L, IL-7, IL-15 and IL-21. After 7-20 days of culture (Stage 3) can obtained both immature NK cells and ILC3. The cytokines production and cytolytic activity increase according to NK cell maturation. After 25-30 days of culture are obtained fully functional mature NK cells ($CD56^{+/-}$CD94/$NKG2a^{+/-}$ LFA-$1^+CD16^+NCR^+NKG2D^+KIRs^+EOMES^+$. (Adapted from Vitale C et al 2012)

1.6 NK cells immunotherapy and Molecular Targeted Therapy

NK cells contribute to the immune response against hematological malignancies such as chronic myeloid leukemia (CML), acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) through NCR-mediated cytotoxic activity and, indirectly, by contributing to the activation of T-CD8 cells and of macrophage responses [58].

The improved cytotoxic activity of NK cells correlates with better prognosis and arrested disease progression. Unfortunately, leukemic blasts can compromise autologous NK cells functions and thus elude the immune-surveillance of the immune system through several tumor escape mechanisms such as: the modulation of NCR/NKG2D ligands, the increase of HLA-I molecules as well as the release of soluble factors [59-62].

The allogeneic-hematopoietic stem cell transplantation (allogeneic-HSCT) is a high-risk treatment for the patients but very effective in eradicating leukemia. Heterologous NK cells derived from HSCT can prevent relapses but not all patients are eligible for this treatment and the effect maybe only transitory, requiring therapy maintenance. For this reason, in the last few years, alloreactive-NK mediated immunotherapy protocols and engineered NK cells (chimeric antigen receptors, CAR) have been designed (see Figure 10) [63-65].

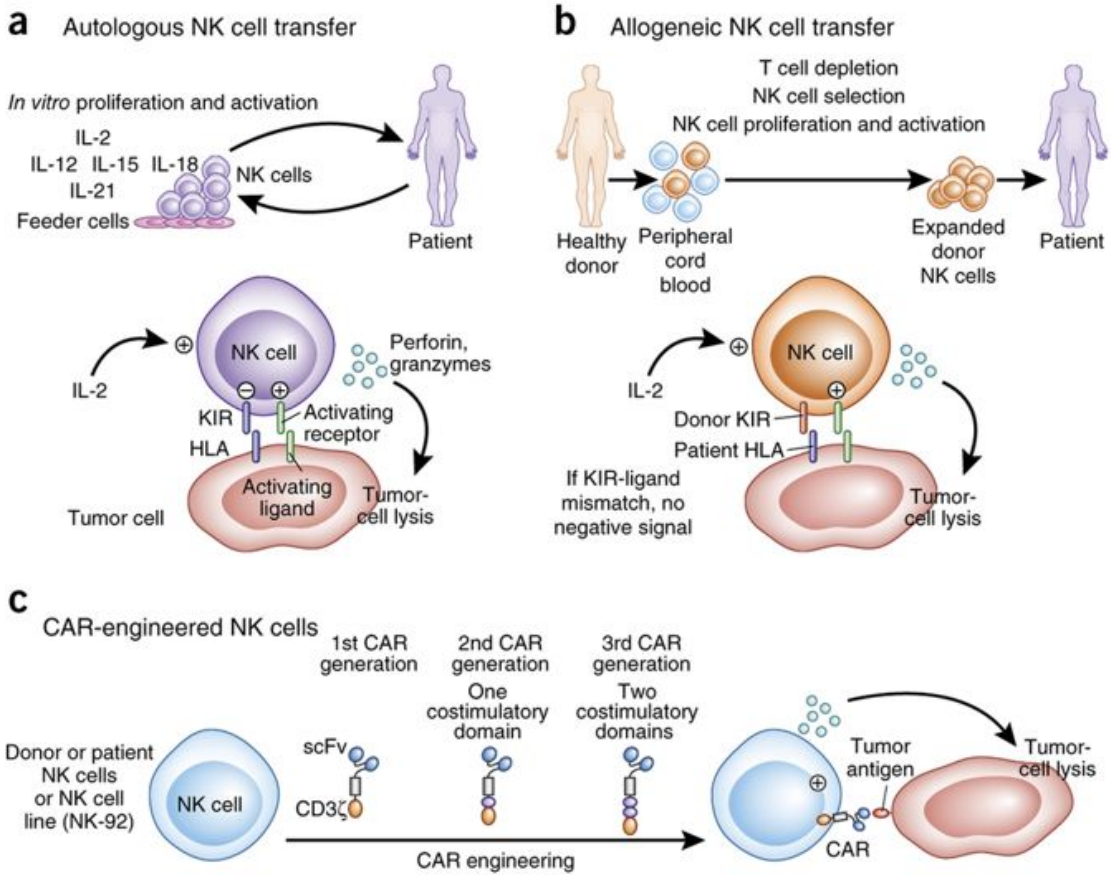

Figure 10. NK cell immunotherapy: a) autologous NK cell are activated and expanded *in vitro* with IL-2, IL-15, IL-21 and IL-18 cytokines and infused into the patient; b) allogeneic NK cells are isolated from CBMC of healthy donor and then are selected. After NK cells activation and proliferation, donor NK cells are infused into the receiving patient; c) patient/donor NK cells or NK cell line (NK92) are engineered with Chimeric Antigen Receptor (CAR) specific for tumor antigens. (Adapted from Guillerey C et al 2016)

Otherwise, the therapy of choice for CML and ALL is still molecular targeting therapy, which has improved the prognosis of patients [66, 67]. Drugs such as selective Tyrosine Kinase Inhibitors (TKIs) have been introduce 20 years ago and had a key role in improving the overall survival of CML patients. However, TKIs cannot be considered as a curative, because high percentages (50-60%) of patients develop resistance or lack of complete molecular remission [66, 68-70].

CML is a myeloproliferative disease that originates from hematopoietic stem cells (HSCs) and is represented by leukocytosis and splenomegaly. CML is characterized by increased proliferation of the granulocytic cell line without the loss of their capacity to differentiate. Consequently, the peripheral blood cell profile shows an increased number of granulocytes and their immature precursors, including occasional blast cells. CML accounts for 20% of all leukemias affecting adults. CML progresses in two phases: a chronic phase (CP), an asymptomatic phase characterized by abnormal proliferation of granulocytes and their precursors in BM and PB involving 90% of patients at diagnosis, and an accelerated phase (AP) or blast crisis, represented by increase numbers of immature leukocytes, similarly to AML, in which symptoms like weight loss, fever, enlarged spleen and anemia begin to appear. In almost all CML patients have been identified the Philadelphia chromosome (Ph) and its corresponding protein BCR-ABL, constitutively activated[71].

BCR-ABL tyrosine kinases are the main target of TKIs, which derive from chimeric gene BCR-ABL, originating from translocation between terminal fragment of chromosome 9 and chromosome 22, that generates the Ph. These aberrant proteins kinases, named p210 (CML and AML associated) and p190 (mainly correlated to ALL), are able to phosphorylate different substrates favoring the proliferation of blasts, reducing apoptosis and modifying adhesion mechanisms and response to regulation factors [72-76].

Imatinib mesylate (STI571, Gleevec, Glivec) was the progenitor drug used in first-line treatment of CML-Ph^+, ALL-Ph^+ and gastrointestinal stromal tumors (GISTs) both in adult and pediatric patients. The targets of Imatinib are BCR-ABL tyrosine-kinase, c-KIT (Stem Cell Factor Receptor), PDGF-R (Platelet-Derived Growth Factor Receptor) and VEGF-R

(Vascular Endothelial Growth Factor Receptor). This therapy presents a lot of side effects; the most common are neutropenia, thrombocytopenia and anemia [77]. Different mechanisms of resistance have been observed, especially in the advanced stage of disease, these include: increases of BCR-ABL kinase through the BCR-ABL gene amplification/mutation (e.g. T315I point mutation), excessive binding of Imatinib to plasmatic proteins, intracellular decrease of drugs due to high expression of Multidrug-resistant P-glycoprotein (or p-glycoprotein 1 coded by MDR1 gene) and high expression of proto-oncogene tyrosine-proteins kinase (Src) [78].

These drug resistances led to the development of second generation TKI that are more potent and selective. Nilotinib (AMN107, Tasigna), that is structurally similar to Imatinib and specific for the same targets, is mainly indicated for the treatment of CML-Ph^+ in adult patients. Dasatinib (Sprycel, BMS-354825) is a BCR-ABL inhibitor that is active on some Src-kinases (Lck and Hck) and also used for the treatment of pediatric patients suffering from LLA-Ph^+. Moreover, Dasatinib is the only drug of TKI family that is capable of crossing the blood-brain barrier (BBB) and acts on brain metastases caused by LLA-Ph^+ [79].

These second generation of inhibitors cannot overcome the resistance due to the presence of T315I point mutation of BCR-ABL. Therefore, new drugs have been developed such as Ponatinib, a third generation TKI approved for the treatment of CML patients that are resistant to, or intolerant to other TKI [67, 72, 77, 80].

The role of TKI therapy in the early-stage of disease control is been demonstrated. On the other hand, the effect of TKI on the outcomes of HSCT in CML patients is under evaluation. A retrospective analysis of 2016 shows that maintenance TKI therapy after HSCT might reduce the incidence of relapse and improve outcomes of high-risk Ph^+ leukemia patients [81].

Clinical evidences have shown that 50% of patients in sustained Deep Molecular Remission (DMR) may interrupt their TKI treatment without clinical relapse (Treatment Free Remission, TFR) [82]. Several experimental and clinical observations supported the notion the TKI may successfully cure leukemia only in those patients in which it is possible to observe the restoration of antitumor immune response [83-85]. In this context, NK cells seem to play an important role in the control of the CML residual disease and this NK cell-mediated immune response may to be correlated with a better clinical outcome [83, 86]. It was observed that in CML patients treated with Imatinib, the increase of cytotoxic mature NK cells was associated with the successful therapy discontinuation [87]. Moreover, it has been reported that Dasatinib induces a rapid $CD56^+CD57^+$ NK cells mobilization in PB and BM of CML patients [88-90]. Dasatinib-treated patients that interrupted therapy and

remained in deep molecular response (DMR) for more than 1 year displayed high numbers of circulating NK cells and CTL [91]. Of note, one of the side effects of Dasatinib treatment is the onset of pleural effusion (PE): the mechanism of Dasatinib-related pleural effusion is still unclear but it has been observed a correlation Dasatinib-mediated lymphocytosis, PE and better clinical outcome [77, 92].

Despite these data, still half of patients relapse after therapy discontinuation: the presence of resistant LSCs (MRD) may be responsible of these events. There are many BCR-ABL independent mechanisms of resistance. LSCs are characterized by low activation/proliferation (state of quiescence), overexpression of p-glycoprotein 1, TGF- α/β , TNF-α, Jak-STAT and reside in BM [72, 78, 93]. Other mechanisms involve signaling pathways such as RAF/MEK/ERK or epigenetic deregulation. Thus, it would be important to analyze the BM microenvironment where leukemic stem cells reside, since it could have a crucial role in promoting disease progression: the surrounding stromal cells such as mesenchymal stromal cell (MSCs) contribute to genesis of leukemic clone and to protection of LSCs from TKIs effects [93, 94]. At the same time, BM microenvironment may interfere with NK cell differentiation, maturation and survival, and therefore impair the eradication of leukemia [95].

	Chronic phase		Accelerated phase	Blastic phase
	First line	Second or later treatment lines		
Imatinib	Approved	After failure of interferon	Approved	Approved
Dasatinib	Approved	Resistance to prior TKIs including imatinib	Approved	Approved
Nilotinib	Approved	Resistance or intolerance to prior therapy including imatinib	Approved	Not approved
Ponatinib	Not approved	Resistance or intolerance to dasatinib or nilotinib and for whom subsequent treatment with imatinib is not clinically appropriate; or with the T315I mutation	Approved	Approved

Figure 1. TKI approved for CML: Imatinib, Dasatinib, Nilotinib are approved both for use in the first line and in the accelerated phase. Ponatinib is not approved in first line but is used in accelerated phase and blastic phase. (Adapted from Garcia-Gutierrez V et al, 2019)

	Imatinib		Dasatinib		Nilotinib		Ponatinib	
	All grades	Grade 3/4	All grades	Grade 3/4	All grades	Grade 3/4	All grades	Grade 3/4
Fatigue	++++	+	+++	+	++++	–	++++	++
Rash	++++	++	+++	+	++++	–	++++	++
Headache	+++	–	++++	–	++++	–	++++	++
Myalgia	+++++	–	++++	–	NR	NR	++++	++
Bone pain	+++	++	NR	NR	NR	NR	NR	NR
Diarrhea	++++	++	++++	+	+++	+	NR	NR
Nausea	++++	–	++++	–	+++	+	++++	+
Vomiting	+++	–	+++	–	++	–	NR	NR
Abdominal pain	++	–	NR	NR	NR	NR	++++	+++
Pancreatitis	+	+	NR	NR	++	++	+++	+++
Peripheral edema	++++	++	++++	++	+++	+	NR	NR
Pleural effusion	++	+	++++	++	++	+	NR	NR
Elevated lipase	++++	+++	NG	–	++++	+++	++++	++++
Hepatotoxicity	++++	++	NG	+	+++++	+++	+++	++
Anemia	+++++	+++	+++++	++++	++++	++	++++	++++
Thrombocytopenia	+++++	++++	+++++	++++	++++	+++	++++	++++
Neutropenia	+++++	++++	+++++	++++	++++	+++	++++	++++

Figure 12. The most common TKI side effects are anemia, neutropenia and thrombocytopenia. Other common side effects are fatigue, rash, headache and nausea. Pleural effusion is more frequently side effect in Dasatinib-treated patients. (Adapted from Garcia-Gutierrez V et al, 2019)

2. AIMS OF THE PROJECT

AIM 1) The first aim of this project was to verify whether the TKI drugs interfere with ILC/NK cells differentiation and modify the phenotype, the production of cytokines and the cytolytic activity of ILC/NK cells. Our goal was to understand whether such compounds may affect the generation of potential antileukemic activity of NK/ILC cells undergoing differentiation in the PB and BM of CML patients. Our results have been published in 2018 (Damele L et al 2018).

Damele, L., Montaldo, E., Moretta, L., Vitale, C., Mingari, M. C. **Effect of Tyrosin Kinase Inhibitors on NK Cell and ILC3 development and function**. *Front. Immunol.* 9, 2433. doi: 10.3389/fimmu.2018.02433 (please see the attached file).

AIM 2) The second aim of my project was to characterize the lymphocyte and myelo-monocyte populations in PB and BM of patients affected by CML before and during treatment with TKI. In particular, I've been focused on the characterization of NK cell and ILC3 populations to integrate data obtained with our *in vitro* model.

3. AIM 1- Effect of Tyrosine Kinase inhibitors on NK cell and ILC3 development and function

Synopsis

Tyrosine kinase inhibitors (TKI) sharply improved the prognosis of Chronic Myeloid Leukemia (CML) and of Philadelphia$^+$ Acute Lymphoblastic Leukemia (Ph$^+$ALL) patients. However, TKI are not curative because of the development of resistance and lack of complete molecular remission in the majority of patients [66-70, 72]. Clinical evidences would support the notion that patient's immune system may play a key role in preventing relapses [82, 83]. In recent years, it was observed that NK cell immune response seems to correlate with a positive clinical outcome [83, 86]. In particular, increased proportions of terminally differentiated CD56$^+$CD16$^+$CD57$^+$ NK cells have been reported to be associated with successful Imatinib therapy discontinuation (TFR) or with a DMR in Dasatinib-treated patients. Thus, it would be important to study whether any TKI have an effect on circulating mature NK cells and on their development by using *in vitro models* that could allow the identification of possible molecular mechanism(s) by which continuous exposure to TKI may influence NK cell development and repertoire [90, 92, 96-98]. To this end, CD34$^+$ hematopoietic stem cells (HSC) were cultured in the absence or in the presence of Imatinib, Nilotinib, or Dasatinib at the plasma peak concentration (to reproduce concentration present in PB and BM of TKI-treated patients), or with DMSO as a vehicle control [56, 95]. Between day 15 and 27 of culture, cells were counted and repeatedly analyzed by flow-cytometry for NK-specific surface markers, cytokines production, cytolytic activity and transcription factors.

The results show that all compounds exert an inhibitory effect on CD56$^+$ cell recovery. In addition, Dasatinib sharply skewed the repertoire of available CD56$^+$ cells towards CD56$^+$CD117$^+$CD94/NKG2a$^-$ROR γ t$^+$ IL-22-producing ILC3 cell population, leading to an impaired recovery of CD56$^+$CD117$^-$CD16$^+$CD94/NKG2a$^+$NKG2D$^+$DNAM1$^+$EOMES$^+$ mature cytotoxic NK cells. Interestingly, the few NK cells undergoing differentiation in the presence of Dasatinib, displayed the capacity to express high percentages of IFN-γ; we also detected a slight increase of IL-8/IFN-γ-producing ILC3. Of note, ILC3 play a crucial role in intestinal inflammation due to their ability to become IFN-γ-producing ILC1 ("ex-ILC3") upon IL-12 stimulation [50-53, 55]. Different from IFN-γ [77, 99], production, both Nilotinib and Dasatinib reduced the cytolytic activity of NK cells undergone *in vitro* differentiation against the leukemia cell line K562.

Further, analysis performed at early time cultures intervals revealed an increase of $CD56^+CD117^+CD127^+$ cells that might represent ILC3 precursors supporting the hypothesis that Dasatinib may skew ILC commitment towards ILC3.

It has been reported that Dasatinib, but not Imatinib and Nilotinib, exerts an inhibitory effect on the family of Src kinases: thus, it was possible that the Dasatinib -mediated effect on ILC differentiation, may reflect an inhibitory effect on Src kinases occurring at the level of cell precursors [79, 100]. To study this, $CD34^+$ cells were cultured with KX2-391 Src-kinase inhibitor used at different concentrations. After 25 days of culture, the cells were collected and analyzed for phenotypic and functional maturation markers. The results showed that the low concentrations of KX2-391 (i.e. 5 μM) did not inhibit $CD56^+$ cells recovery. However, similarly to the Dasatinib-mediated effect, the use of this inhibitor increased the expression of CD117 and RORγt TF and decreased the expression of CD94/NKG2a and EOMES TF. Moreover, the $CD56^+$ cells undergoing differentiation in the presence of KX2-391 displayed a low cytotoxic activity similar to Dasatinib-treated cells.

Previous studies on *in vitro* human ILC differentiation have shown that also the cytokines used in this model of NK cell differentiation may play a role in the ILC commitment. In particular, SCF has a key role in ILC3 development in the presence of IL-7 or IL-15, while IL-15 and IL-7 alone, or in combination with other cytokines such as IL-1β, skew differentiation towards $CD56^+$ NK cells [47, 95, 100, 101]. In this context, it has been described that IL-7 is required for ILC3 homeostasis and development within the lymph nodes [102]. IL-7 exploits both STAT3 and STAT5 transduction pathway; IL-15 preferentially uses STAT5 signaling protein while the interaction between SCF and c-KIT (CD117) involves STAT3 protein. Of note, it has been observed by Konig H et al. that Dasatinib, transiently inhibits STAT3 phosphorylation, while induced a durable inhibition of STAT5 signaling pathways in $CD34^+$ cells isolated from CML patients at diagnosis [79]. This durable inhibition seems to be due to the inhibitory effects mediated by Dasatinib on Src-kinase activation pathway that, in turn, modulate STAT5 phosphorylation [100, 103]. Our analyses of STAT3/STAT5 phosphorylation status in Dasatinib–stimulated $CD34^+$HSC revealed a more durable reduction of $pSTAT5^+$ cells as compared to $pSTAT3^+$ cells during the first days of culture. These results, according to the early expression of CD117 and CD127 observed on Dasatinib-treated precursors, may support the hypothesis that the the SCF/c-KIT and IL-7/CD127-mediated STAT3 transduction pathway, may overcome Dasatinib-mediated inhibition and favor the preferential ILC3 precursors survival and maturation.

It has been observed that Dasatinib may induce lymphocyte's mobilization that may lead to systemic inflammation and side effects such as pleural effusion and colitis [77, 90, 92]. This could be associated with the increased of NK and IFN-γ or IL-22 producing ILC3/ILC1. On the other hand, our data are in contrast with *in vivo* data that documented the presence of cytotoxic NK cells *in vivo* in the PB and the BM of Dasatinib-treated patients, maybe because to a high stability of the drug in our cell cultures. It has been suggested that the numbers of circulating cytotoxic NK cells and cytotoxic T lymphocytes (CTL) were significantly higher in DMR patients [88, 89]. The high degree of heterogeneity of analyzed cases may contribute to the variability of responses: patients treated with different TKIs, and the immune system status (e.g. viral activation) may influence immune cell repertoire and function and then the clinical outcome of CML-patient.

Taken together, our studies reveal a possible mechanism by which Dasatinib may interfere with the proliferation and maturation of fully competent NK cells, i.e.by targeting signaling pathways required for differentiation and survival of NK cells but not of ILC3.

Finally, our study could identify new tools to design individualized timing and dosing of Dasatinib application, in order to obtain good responses without impairment of NK cell-based immunotherapeutic interventions.

Ref: **Damele, L**., Montaldo, E., Moretta, L., Vitale, C., Mingari, M. C. **Effect of Tyrosin Kinase Inhibitors on NK Cell and ILC3 development and function**. *Front. Immunol.* 9, 2433. doi: 10.3389/fimmu.2018.02433

4. AIM 2- Analysis of the NK cell repertoire in CML patients undergoing TKI therapy.

4.1 Introduction

As discussed previously, in the last years, several papers reported data on the NK cell repertoire in CML patients undergoing TKI therapy. Generally, these papers support a correlation between the achievement of a durable DMR, also in the case of TKI therapy suspension, with the presence of mature $CD56^{+}CD57^{+}KIR^{+}NKG2a^{-}$ cytotoxic NK cells in patients' peripheral blood. However, in view of these interesting findings, more detailed analysis could further contribute to identify patients potentially eligible for a safe therapy suspension. In particular, patient's longitudinal analysis and comparison between patient's PB- and BM-derived NK cells could offer new insights in the potential ability of these cells to eradicate residual leukemic disease.

In view of the in vitro data on NK /ILC differentiation in the presence of TKI drugs that we previous reported, we decided to start a longitudinal follow up of CML patients undergoing TKI therapy at UO Hematology 1 and Hematological clinic of San Martino Polyclinic Hospital in Genoa. Our plan is to perform deep analyses that should include characterization of NK and T cell, of myeloid cell compartment, and of soluble plasma-derived factors. Below, we present very preliminary data regarding NK cells obtained in the first 5 patients that we could analyze.

4.2. RESULTS

4.2.1 Analyses of PB NK cells in patients undergoing TKI therapy

The lymphoid cell repertoire of five patients affected by CML was analyzed at diagnosis and at different time intervals during TKI therapy (1-3-6-12 months). Below, we present data regarding four patients undergoing Nilotinib therapy and one patient undergoing Dasatinib treatment. For further patients details please see **Table 1** and Materials and Methods section.

We focused our studies on PB NK cell repertoire: mononucleated cells were freshly isolated from PB and subjected to flow cytometric analyses for the identification of the specific surface markers (**Figure 13**). The results indicated that Nilotinib patients have lower $CD3^+$ cell percentages at diagnosis as compared to other time intervals during therapy, while the percentages of $CD56^+$ cells remain quite stable in time (**Figure 13B**). The only patient undergoing Dasatinib treatment showed a decrease of $CD3^-CD56^+$ NK cell percentages during the first month of therapy, while the percentages of $CD3^+$ cells increased during therapy. Among $CD56^+$ cell subsets, the percentages of $CD56^{bright}$, including $CD117^+$ cells, were more represented at diagnosis while decreased during therapy, in particular in Nilotinib-treated patients. On the other hand, the percentages of terminally differentiated $CD56^+CD57^+$ NK cells increased during both Nilotinib and Dasatinib therapy treatment. In particular, Nilotinib-treated patients displayed higher percentages of $CD56^+CD57^+$ cells as compared to the Dasatinib-treated patient (**Figure 13C**). The further analyses indicated that both in the presence of Dasatinib and Nilotinib there was a high variability of $CD56^+$ NK cell percentages expressing activating receptors during the different time intervals. Of note, despite the high variability of NK cell percentages expressing NKp46 and NKG2D receptors, we could observed a progressive increase of NKp46 and of NKG2D surface MFI on $CD56^+$ cells in Nilotinib-treated patients (**Figure 14A**, **Figure 15**). On the other hand, Dasatinib-treated patient displayed an increase of $CD56^+NKG2D^+$ cells during therapy as compared to diagnosis, but the MFI greatly varied among the different time intervals. In the same patient, $NKp30^+$ NK cells decreased after 6 months of therapy, while the receptor MFI maintained higher levels as compared to diagnosis. Finally, $DNAM\text{-}1^+$ cells could be always observed at different time intervals both in Nilotinib and Dasatinib patients however, in Nilotinib patients' receptor MFI rapidly increased during therapy, while in Dasatinib patient receptor MFI increased only after one year of therapy (**Figure 14A, Figure 15**). The detection of NK inhibitory receptors revealed that the percentages of $CD56^+CD94/NKG2a^+$ were more represented in Dasatinib-treated patient as compared to Nilotinib-treated patients. In particular, the patient

displayed high percentages of $CD56^+CD94/NKG2a^+$ that decreased only after 12 months from the beginning of the therapy. Of note, we always detect high percentages of $CD94/NKG2c^+$ NK cells in Nilotinib patients at all time intervals analyzed. We know analyzed in Nilotinib patients. In this context we know that none of these patients suffered of CMV reactivation or infection during therapy but we could obtain data on CMV positive serology at diagnosis for only 2/4 of the Nilotinib patients (**Figure 14B**).

In order to characterize the functional features of NK cells, we evaluated cytokine production and the $CD56^+$ cells cytotoxic potential by the CD107a degranulation assay. **Figure 14C,** shows $CD107a^+$ NK cell percentages detectable after 3 hours of incubation with the HLA-class I^- K562 leukemic cell line. Both Nilotinib-treated patients and Dasatinib-treated patient displayed an increase of $CD107a^+$ cells during therapy as compared to the diagnosis. However, our preliminary data indicated that Nilotinib-treated patients experienced a decrease of $CD107a^+$ cells at 12 months while, at the same time interval, Dasatinib-treated patient still displayed a 40% of $CD107a^+$ NK cells upon incubation with K562 cells. As shown in **Figure 14C**, unfortunately we could not perform $CD107^+$ analysis on Dasatinib patient at diagnosis thus, the histograms represent data starting from 1 month after beginning of the therapy.

To test intra-cytoplasmic cytokines production, patient-derived PB lymphocytes were overnight stimulated with IL-12, IL-15 and IL-18 and analyzed for the expression of IFN-γ by flow cytometer analysis. The results revealed that $CD56^+$ cells derived both from Nilotinib- and Dasatinib-treated patients could express similar percentages of $CD56^+IFN\text{-}\gamma^+$ cells and these amounts were also comparable to those observed in healthy controls (**Figure 14D**). As shown in **Figure 14D**, unfortunately we could not perform cytokine expression analysis on Dasatinib patient at diagnosis thus, the histograms represent data starting from 1 month after beginning of the therapy.

Our *in vitro* data on NK cell differentiation would suggest that Dasatinib treatment could favor the development of ILC3 that displayed higher percentages of $IL\text{-}22^+$-producing $CD56^+$ cells as compared to controls. Thus, we verified the ability of lymphocytes isolated from TKI treated patients to produce IL-22. To this end, lymphocytes were stimulated also with IL-1β, IL-7, and IL-23 cytokines, known for their ability to induce IL-22 production by innate lymphoid cells. Again, we could not perform such analysis in Dasatinib-treated patient at diagnosis. Our results would suggest that Dasatinib-treated patient displayed higher percentages of $CD56^+$ cells able to express detectable amounts of IL-22 as compared to Nilotinib patients (**Figure 14D**).

4.2.2 Dasatinib-treated patient displayed detectable amounts of ILC3 RORγt$^+$IL-22 producing cells in the peripheral blood at early time intervals following therapy beginning.

In view of our *in vitro* data on NK/ILC development in the presence of TKI, we also evaluated the presence of ILC3 in the PB of our patients. To this end, PB ILC cell percentages (CD45$^+$CD3$^-$CD14$^-$CD19$^-$CD127$^+$) and the expression of ILC3 lineage-related RORγt TF and IL-22 cytokine production were evaluated by flow cytometry (**Figure 16A**). The **Figure 16B** shows that very low percentages of ILCs were detectable in patients PB. Interestingly, our preliminary data revealed that Dasatinib-treated patient express little higher percentages of ILCs at 1 and 3 months as compared to Nilotinib-treated patients. At the same time intervals, Dasatinib-treated patient displayed higher percentages of LIN$^-$ CD127$^+$CD117$^+$RORγt$^+$ cells as compared to Nilotinib patients and to healthy controls. Functional analyses revealed that RORγt TF expression paralleled the expression of IL-22, meaning that, Dasatinib-treated patient had higher percentages of LIN$^-$ CD127$^+$CD117$^+$IL-22$^+$ cells as compared to Nilotinib patients and to healthy controls (**Figure 16C**).

4.2.3 BM-derived NK cells in CML patients at diagnosis are enriched in CD56^{bright} CD16$^-$ cells and display lower levels of activating receptors and lower ability to express IFN-γ .

We could also analyze the medullary blood of 3/4 Nilotinib-treated patients that we received at diagnosis and after three months of therapy. The **Figure 17A** shows that the percentages of CD3$^+$ and CD56$^+$ cells in PB were higher than in BM at diagnosis. At diagnosis, in the BM the percentages of CD56^{brigh} cells detectable in BM, including CD117$^+$cells, slightly overcome CD56^{dim} CD56$^+$CD57$^+$ cells, which, on the other hand, represented the majority of NK cells in the PB (**Figure 17B**). Analyses of NK cell receptor expression revealed that in the BM the median percentages of CD56$^+$cells expressing NKp30, NKG2D and DNAM-1 were lower than those detected in the PB. Of note, we could also observe that NKp30 MFI decreased after 3 months, in particular in the BM (**Figure 18A, Figure 19**). We also compared the expression of inhibitory receptors on CD56$^+$ NK cells in PB and in BM. Data are limited, however, it seems there could be a different distribution of NK cell subsets between PB and BM (**Figure 18B**). In particular, it seems that at diagnosis CD56$^+$cells present in the BM expressed low proportion of KIR

receptors. The analyses of CD107a expression upon incubation with K562 cell line revealed that during Nilotinib therapy a sharp increase of $CD56^{+}CD107a^{+}$ percentages both in PB and BM could be observed (**Figure 18C**). Finally, **Figure 18D** shows that BM-derived NK cells expressed lower amounts of IFN-γ upon stimulation with IL-12, IL-15 and IL-18 as compared to PB-derived NK cells. Notably, the percentages of BM-derived $CD56^{+}IFN\gamma^{+}$ cells increased during therapy either in the BM either in the PB. Finally, we observed that $CD56^{+}IL\text{-}22^{+}$ cells were more represented in BM as compared to PB.

4.3. FIGURES AND TABLE

Table 1-

Patient	Disease	Age at Diagnosis	Sex	Therapy	BCR-ABL1 Post 1 m	BCR-ABL1 Post 3 m	BCR-ABL1 Post 6 m	BCR-ABL1 Post 9 m
STKI001	CML-CP	31	M	NIL	ND	0,128%	0,028%	0,015%
STKI003	CML-CP	43	F	DAS	71,5 %	1,059 %	0,795 %	0,657 %
STKI004	CML-CP	27	M	NIL	9,9%	0,243%	0,035%	0,032%
STKI005	CML-CP	36	M	NIL	21%	0,42%	0,058%	0,059%
STKI006	CML-CP	50	M	IM-NIL	71% (1 M Nil 46%)	1,39%	1,38%	0,886%

The patients' samples were collected by the hematology clinic at the San Martino Polyclinic Hospital.
CML-CP= Chronic Myeloid Leukemia- Chronic Phase
m= month(s)
M= Male, F= Female, IM= Imatinib, NIL= Nilotinib, DAS= Dasatinib
% Of p210 copies
<0,1% BCR-ABL1--> MMR; <0,01% BCR-ABL1--> DMR

Table 1: This Table shows the reverse transcription quantitative polymerase chain reaction (RTqPCR) of BCR-ABL1 mRNA levels of patients affected by CML at different time intervals after therapy. The percentages refer to the contents of p210 copies in patients' PB. This is a technique to evaluate if the patients responded to treatments and in some cases to indicate the need to change or reassessed therapy.

Figure 13-

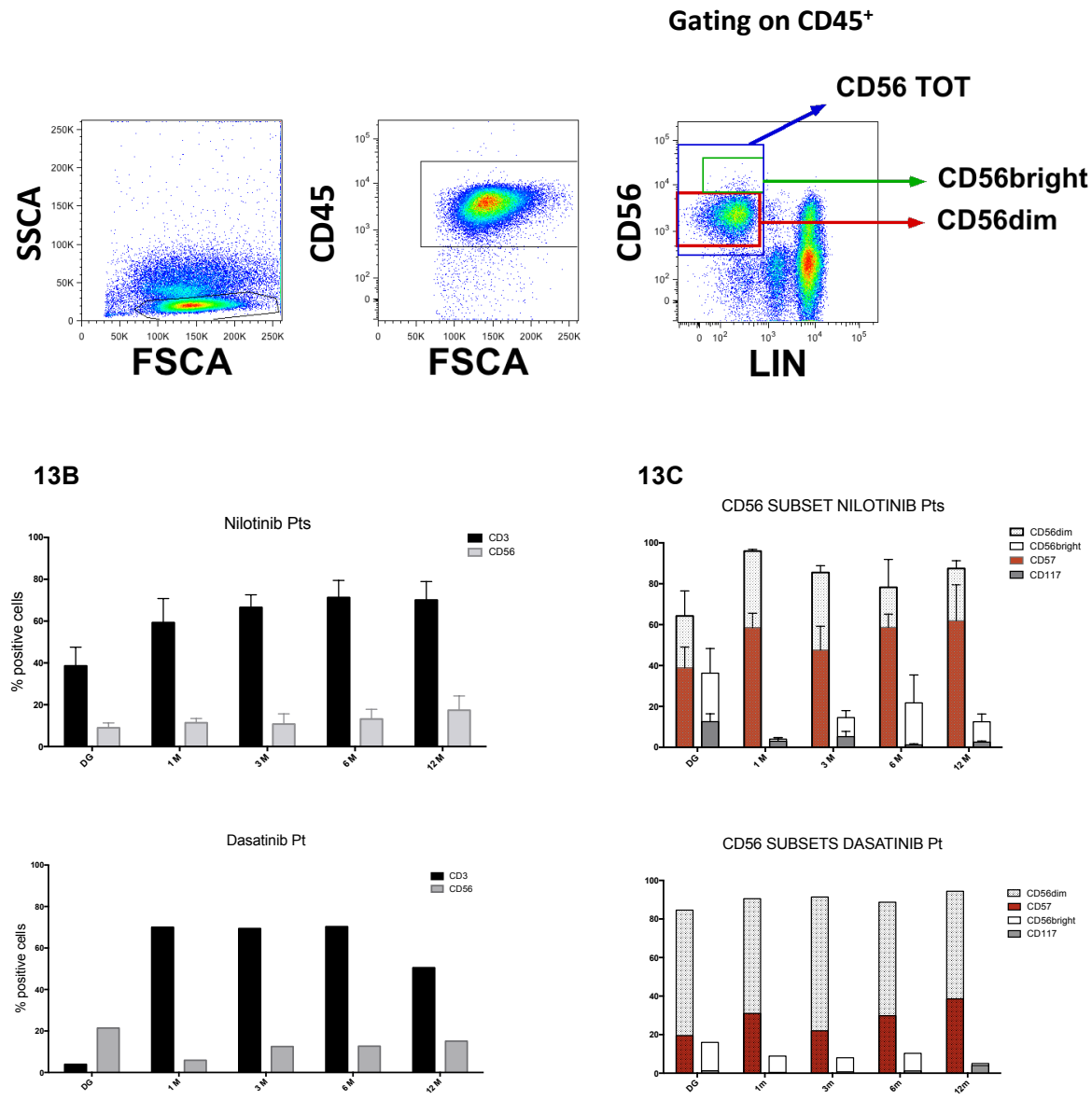

Figure 13: Fresh PBMC have been isolated from peripheral blood of CML patients at diagnosis and at different time points after the start of therapy (1 month, 3 months, 6 months and 12 months) and analyzed by flow-cytometry for the indicated surface markers. **13A**) Dot plots show the gating strategy used to identify $CD56^{+}$ TOT, $CD56^{dim}$ and $CD56^{bright}$. $CD56^{+}$ cells were identified by the absence of lineage (CD3, CD14, CD19). **13B)** The histograms show the percentages of $CD3^{+}$ and $CD56^{+}$ cells in Nilotinib-treated patients and in the only one patient treated with Dasatinib. The data related to Nilotinib-treated patients are represented as the median with interquartile range. **13C)** The histograms show the percentages of $CD56^{dim}CD16^{+}$, $CD56^{bright}CD16^{-}$, $CD56^{+}CD57^{+}$ and

$CD56^{+}CD117^{+}$ cells in Nilotinib-treated patients and Dasatinib-treated patient. The data related to Nilotinib-treated patients are represented as the median with interquartile range.

Figure 14-

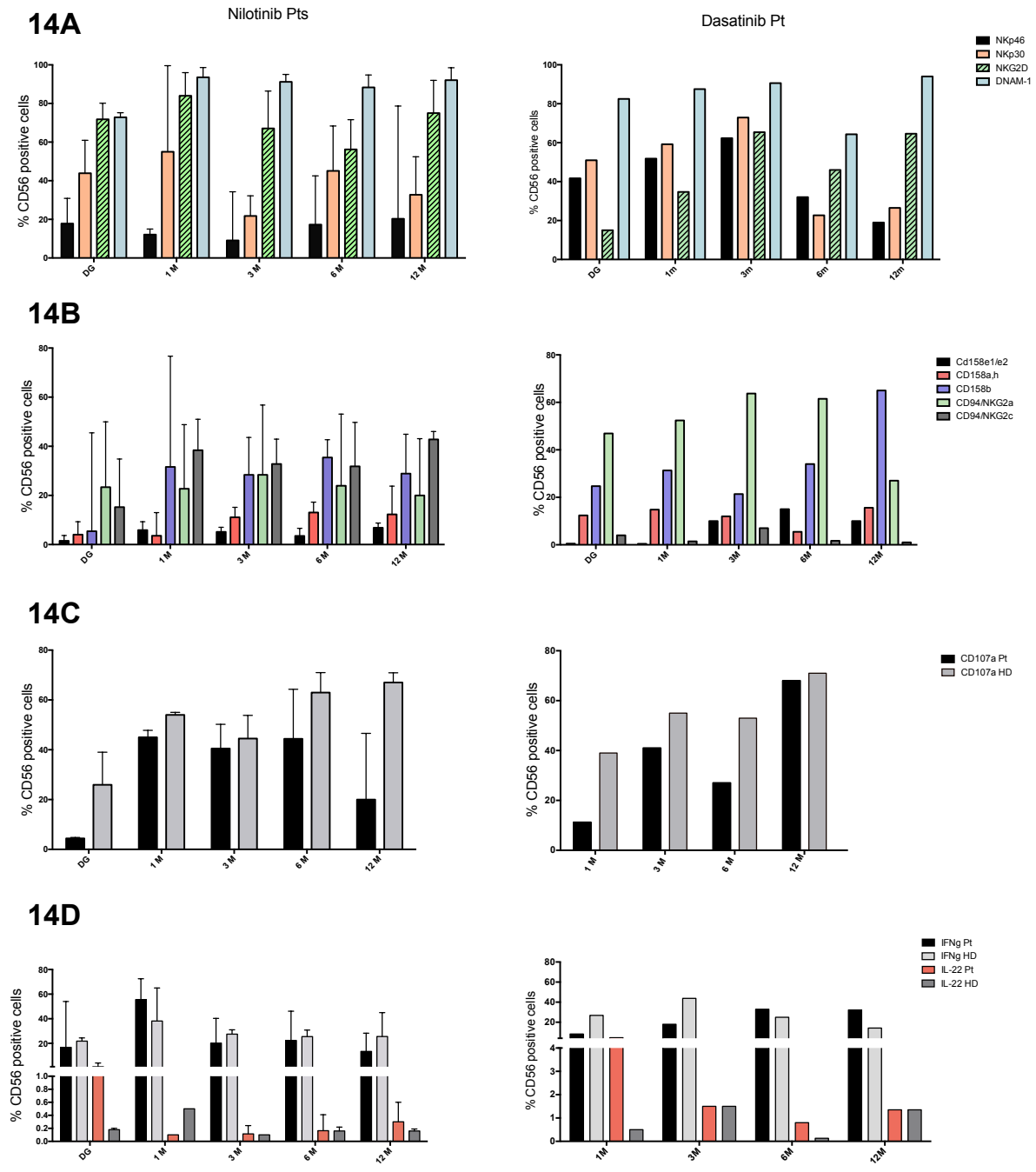

Figure 14: Fresh PBMC have been isolated from peripheral blood of CML patients at diagnosis and at different time points after the start of therapy (1 month, 3 months, 6 months and 12 months) and analyzed by flow-cytometry for the indicated surface markers, transcription factors (TF), CD107a expression and cytokines production. **14A)** The histograms show the percentages of $CD56^{+}NKG2D^{+}$, $CD56^{+}DNAM\text{-}1^{+}$, $CD56^{+}NKp30^{+}$ and $CD56^{+}NKp46^{+}$. The data related to Nilotinib-treated patients are represented as the median with interquartile range. **14B**) The histograms show the percentages of

$CD56^+CD158e1/e1^+$ (KIR3DL1, DS1), $CD56^+CD158a,h^+$ (KIR2DL1, DS1), $CD56^+CD158b^+$ (KIR2DL2,DL3,DS2), $CD56^+CD94/NKG2a^+$ and $CD56^+CD94/NKG2c^+$ cells of Nilotinib-treated patients and Dasatinib-treated patient. The data related to Nilotinib-treated patients are represented as the median with interquartile range. **14C)** The histograms show the percentages of $CD56^+CD107a_+$ of Nilotinib-treated patients and Dasatinib-treated patient compared to healthy donors. The data related to Nilotinib-treated patients are represented as the median with interquartile range. **14D)** The histograms show the percentages of $CD56^+IFN\text{-}\gamma^+$ and $CD56^+IL\text{-}22^+$ of Nilotinib-treated patients and Dasatinib-treated patient compared to healthy donors. The data related to Nilotinib-treated patients are represented as the median with interquartile range.

Figure 15-

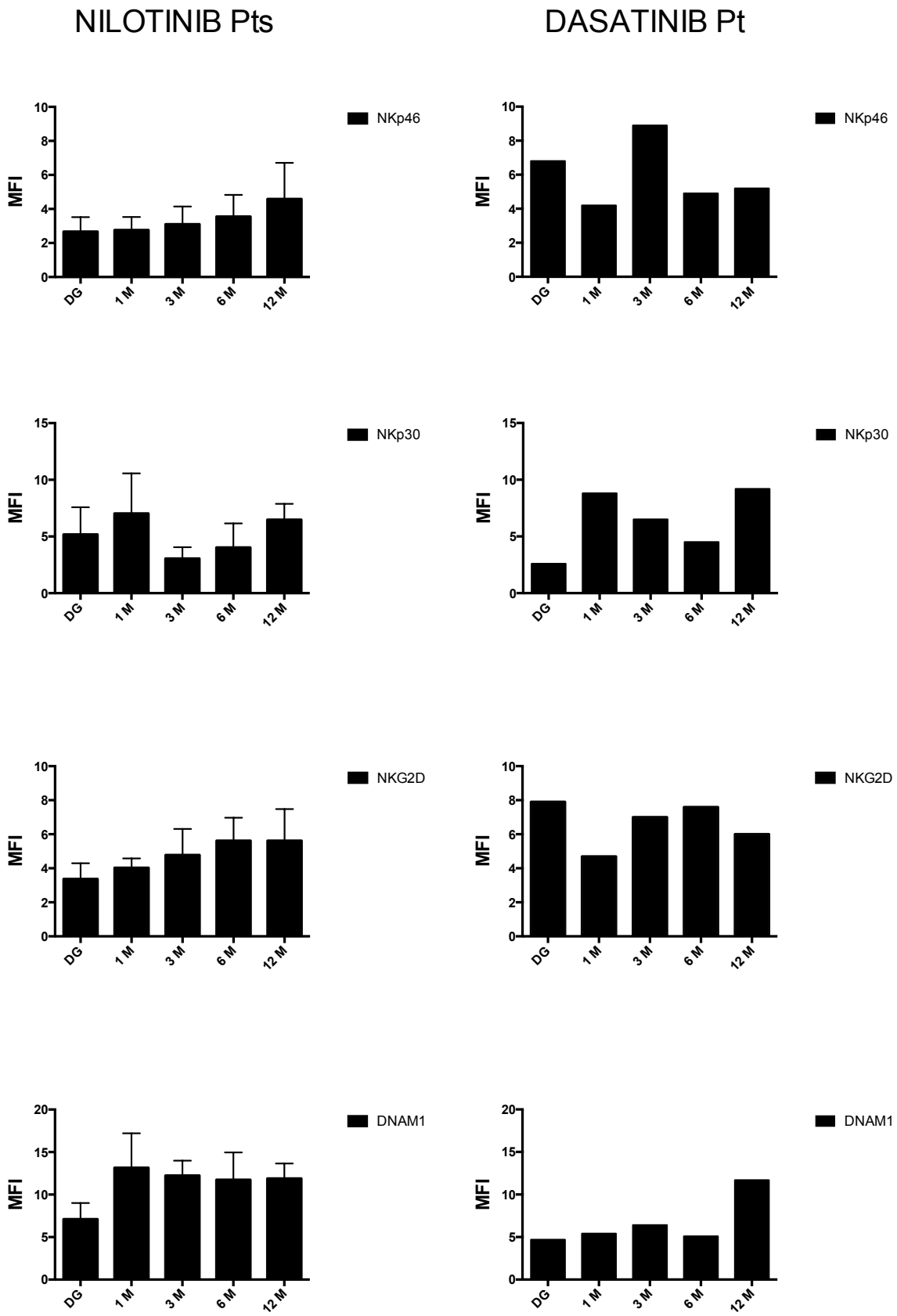
NILOTINIB Pts
DASATINIB Pt
NKp46
NKp30
NKG2D
DNAM1
MFI
DG
1 M
3 M
6 M
12 M

Figure 15: Expression of NKp46, NKp30, NKG2D and DNAM-1 $CD56^{+}$NK cells activating receptors in Dasatinib- and Nilotinib-treated patients' PB at diagnosis and at different time intervals during therapy. MFI was analyzed by flow cytometry (Mean Positive - Mean Negative/2 Negative SD). The data are represented as the median with interquartile range.

Figure 16-

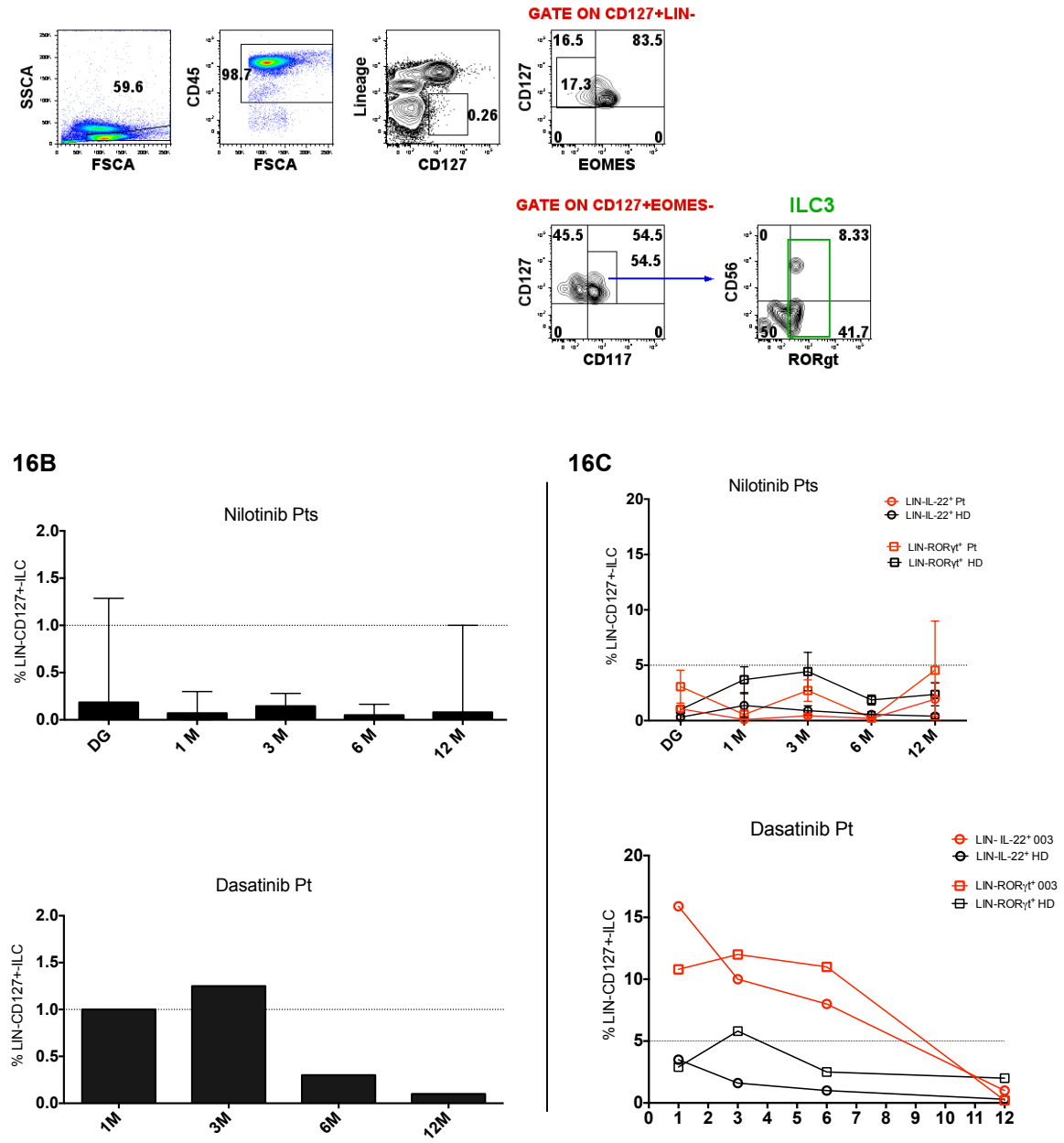

Figure 16: Fresh PBMC have been isolated from peripheral blood of CML patients at diagnosis and at different time points after the start of therapy (1 month, 3 months, 6 months and 12 months) and analyzed by flow-cytometry for the indicated surface markers, RORγt TF and IL-22 cytokine production. **16A**) Dot plots show the gating strategy for ILC3 detection. ILC3s were identified by the absence of lineage (CD3, CD14, CD19) and the expression of CD127, CD117 and RORγt markers. **16B)** The histograms show the percentages of $LIN^{-}CD127^{+}$-ILC cells of Nilotinib-treated patients and Dasatinib-treated patient**.** The data related to Nilotinib-treated patients are represented as the median with

interquartile range. **16C)** The histograms show the percentages of RORγt^{+} cells IL-22^{+} cells gated on CD45^{+}LIN^{-}CD127^{+}CD117^{+}EOMES^{-} cells detected in Nilotinib-treated patients and Dasatinib-treated patient compared to healthy donors. The data related to Nilotinib-treated patients are represented as the median with interquartile range.

Figure 17-

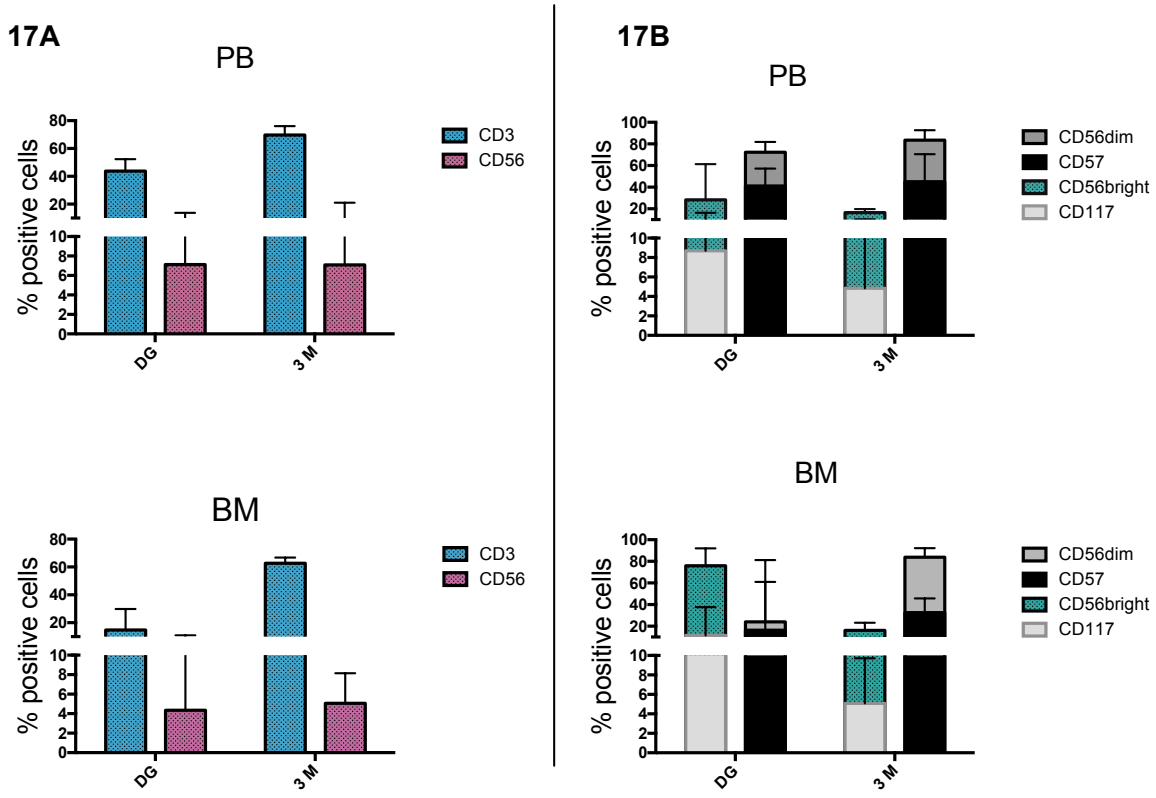

Figure 17: Fresh mononuclear cells have been isolated from peripheral blood and bone marrow of CML patients at diagnosis and after three months from the beginning of Nilotinib therapy and analyzed by flow-cytometry for the indicated surface markers. **17A)** The histograms show the percentages of $CD3^+$ and $CD56^+$ cells in PB and BM of CML patients. The data are represented as the median with interquartile range. **17B)** The histograms show the percentages of $CD56^{dim}CD16^+$, $CD56^{bright}CD16^-$, $CD56^+CD57^+$ and $CD56^+CD117^+$ cells in PB and BM of CML patients.

Figure 18-

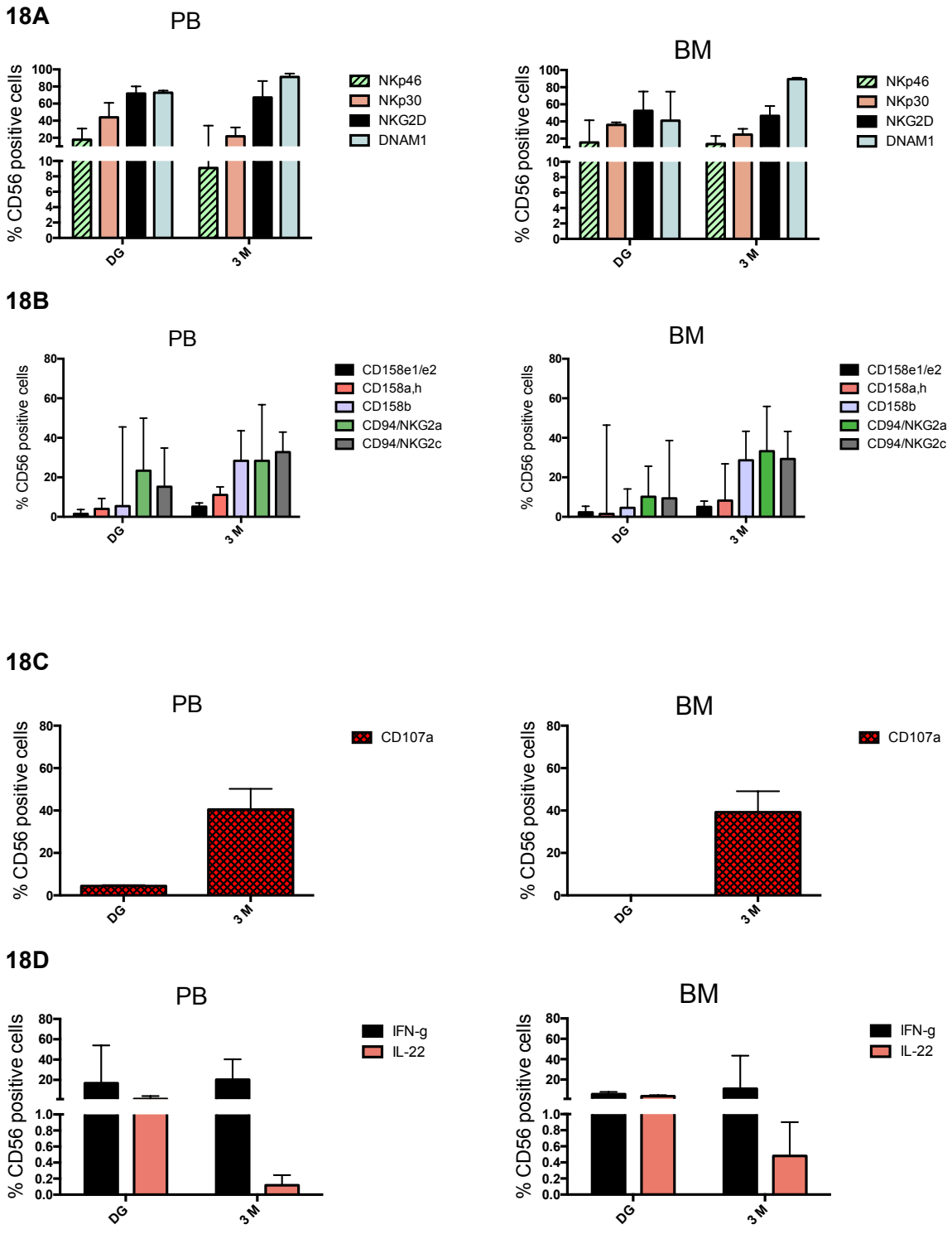

Figure 18: Fresh mononuclear cells have been isolated from peripheral blood and bone marrow of CML patients at diagnosis and after three months from the beginning of Nilotinib therapy and analyzed by flow-cytometry for the indicated surface markers, CD107a expression and cytokines production. **18A)** The histograms show the percentages of $CD56^{+}NKG2D^{+}$, $CD56^{+}DNAM\text{-}1^{+}$, $CD56^{+}NKp30^{+}$ and $CD56^{+}NKp46^{+}$ in PB and BM of CML patients. The data are represented as the median with interquartile range. **18B)**

The histograms show the percentages of $CD56^+CD158e1/e1^+$ (KIR3DL1, DS1), $CD56^+CD158a,h^+$ (KIR2DL1, DS1), $CD56^+CD158b^+$ (KIR2DL2,DL3,DS2), $CD56^+CD94/NKG2a^+$ and $CD56^+CD94/NKG2c^+$ cells in PB and BM of CML patients **18C)** The histograms show the percentages of $CD56^+CD107a^+$ in PB and BM of CML patients. The data are represented as the median with interquartile range. **18 D)** The histograms show the percentages of $CD56^+IFN\text{-}\gamma^+$ and $CD56^+IL\text{-}22^+$ in PB and BM of CML patients. The data are represented as the median with interquartile range.

Figure 19-

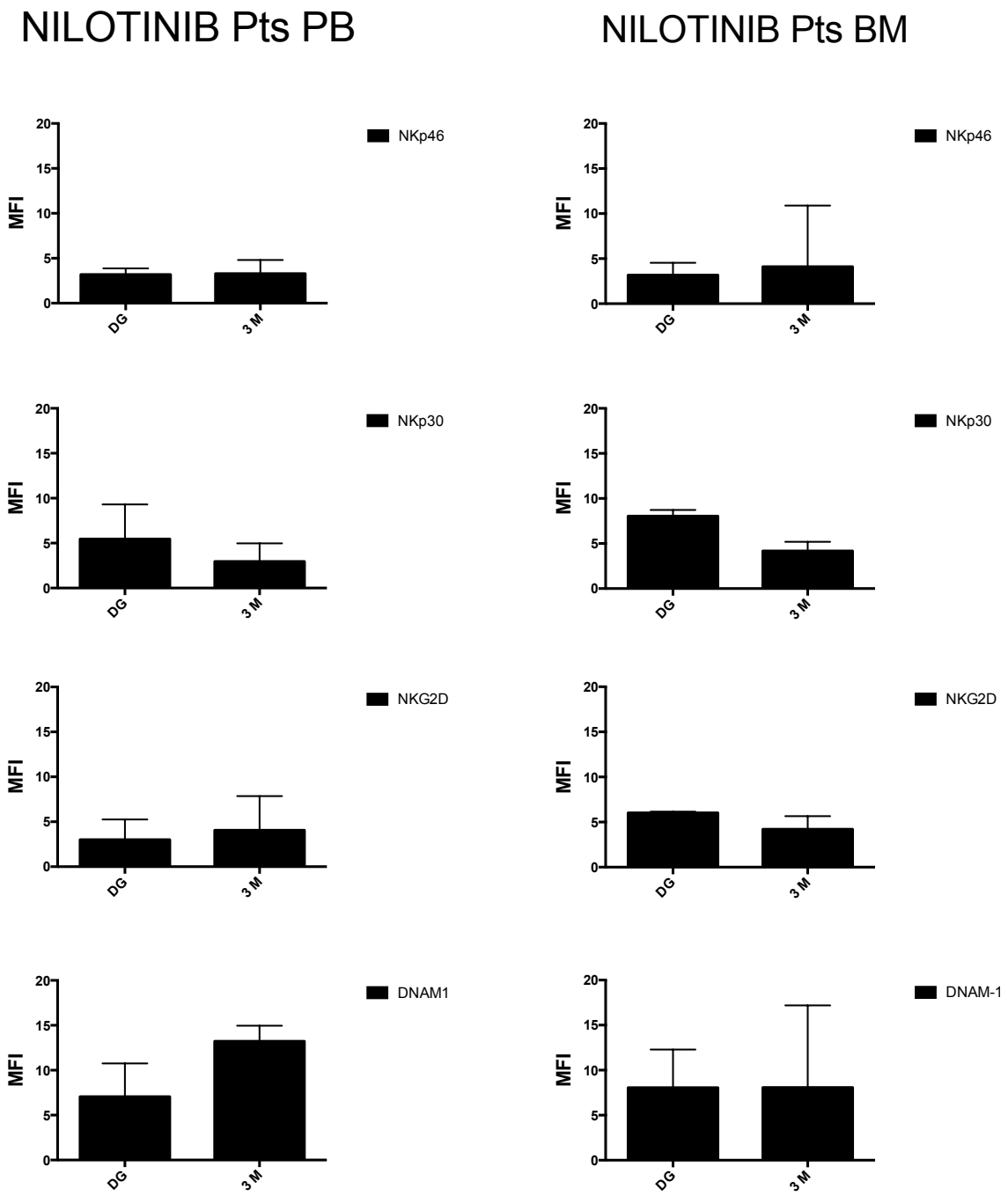

Figure 19: Expression of NKp46, NKp30, NKG2D and DNAM-1 $CD56^{+}$NK cells activating receptors in Nilotinib-treated patients' PB and BM at diagnosis and after three months from the beginning of Nilotinib therapy. MFI was analyzed by flow cytometry (Mean Positive - Mean Negative/2 Negative SD). The data are represented as the median with interquartile range.

4.4. DISCUSSION

The second aim of my project was dedicated to the characterization of immune cell repertoire in CML patients undergoing TKI therapy. In view of our data obtained with the *in vitro* model of NK cell /ILC differentiation, we present and discuss our preliminary data regarding NK cells and ILC3, obtained in the five patients analyzed at diagnosis and during the first year of therapy.

Overall, TKI therapy rapidly restored high percentages of circulating T lymphocytes while the percentages of NK cells did not significantly change. Analyses of NK cell major subsets indicated that $CD56^{bright}$ decreased after beginning of TKI therapy and an increase of $CD56^{dim}CD16^{+}CD57^{+}$ cells could be observed, particularly in Nilotinib-treated patients. It is important to remind that high percentages of $CD56^{dim}CD16^{+}CD57^{+}$ cells have been correlated with a better molecular response in Imatinib and in Dasatinib-treated patients, while high percentages of $CD56^{bright}$ would correlate with a rapid molecular relapse [87, 88]. The expression of activating receptors on $CD56^{+}$ NK cells in Nilotinib- and Dasatinib-treated patients displayed high variability during therapy. Dasatinib-treated patient presented very low percentages of NKG2D at diagnosis, however its expression increased during therapy treatment. In the same patient a major decrease of $NKp30^{+}$ NK cells was observed at last time intervals paralleling the surface expression stabilization of NKG2D and DNAM-1 receptors. The expression of CD94/NKG2a on $CD56^{+}$NK cells in Dasatinib treated patient was higher than in Nilotinib-treated patients. Of note, $CD56^{+}CD94/NKG2a^{+}$ cell percentages in Dasatinib-treated patient decreased after 12 months and remained at low percentages also in further analyses performed at 18 months of therapy (data not shown): at the same time intervals, the percentages of $CD56^{+}KIR2DL2/DL3/DS2^{+}$ cell subset increased in the same Dasatinib-treated patient. Recently, in large cohort of CML patients, it has been shown that Dasatinib treatment induced a decrease of $CD56^{+}NKG2a^{+}$ NK cells that paralleled a higher NK cell cytotoxicity as compared to patients undergoing Imatinib and Nilotinib treatment [104]. The evaluation of degranulation capability of NK cells subset revealed that both TKI drugs increased the percentages of NK cells expressing $CD107a^{+}$ cells upon incubation with K562 leukemia cell line as compared to diagnosis. However, Nilotinib-derived $CD56^{+}$cells displayed a reduction of degranulation capability after one year of therapy that may parallel the reduction of cytolytic activity that we observed in NK cells undergoing *in vitro* differentiation in the presence of this compound[105]. On the other hand, Dasatinib-treated patient still displayed $CD56^{+}$cells with a good degranulation capability at 12 months. These data would confirm that Dasatinib treatment might maintain stable NK cells cytotoxic activity against CML, also thank to a

good expression of activating receptors (i.e.NKG2D) and decreased expression of CD94/NKG2a inhibitory receptor on NK cells at the same interval. According to literature, the TKI-treated patients with high numbers of mature cytotoxic NK cells could be eligible to interrupt TKI therapy and achieved TFR [87, 91, 106, 107]. We observed that $CD56^+$ NK cells developed in the presence of Dasatinib did not express CD94/NKG2a but displayed a reduced cytolytic activity in our *in vitro* NK/ILC differentiation studies [105]. The different Dasatinib-mediated effect on NK cells cytotoxic activity detectable *in vitro* and *in vivo* may be related to short half-life of the drug in plasma patients vs. its durable and prolonged drug stimulation and/or cell activation status in *in vitro* experiments.

Analyses of IFN-γ production by NK cells isolated from patient's PB suggested that TKI-treated patients displayed increased proportions of IFN-γ^+ cells upon therapy initiation, and IFN-γ expression remains stable during therapy both in Nilotinib- and Dasatinib-treated patients and was comparable to healthy controls. These data are in agreement with those obtained in our *in vitro* experiments showing that IFN-γ production was not affected but rather improved by TKI treatment[105]. Accordingly, our results also suggest that Dasatinib favored the production of IL-22 by $CD56^+$ cells after the beginning of therapy. Our analyses for the identification of ILC helper subsets in PB revealed that Nilotinib patients displayed very low amounts of LIN^-CD127^+ cells. Of note, only Dasatinib-treated patient displayed clearly detectable percentages of LIN^-CD127^+. These cells also expressed higher percentages of $LIN^-CD117^+ROR\gamma t^+$ cells as compared to Nilotinib-treated patients until 6 months since the start of therapy. These data paralleled the higher percentages of $LIN^-CD127^+CD117^+IL\text{-}22^+$ cells detected in Dasatinib-treated patient as compared to Nilotinib-treated patients at the same times intervals. These preliminary results might represent a positive feedback of our data on Dasatinib-mediated effects on *in vitro* NK/ILC differentiation, suggesting a positive role of Dasatinib on ILC3 enrichment[105].

Recently, IL-22/IL-17 producing ILC3 have been detected in high percentages in malignant pleura effusion compared to the other ILC subtypes [108]. Of note, it has been observed that pleural effusions are the more frequent side effect in Dasatinib-treated patients as compared to other TKI drugs [77]. It has been reported that patients experiencing lymphocytosis during Dasatinib treatment had higher rates of PE that were also associated with improved response [92]. We could analyze only one Dasatinib-treated patient (STKI003), who displayed low level of ILC3 and did not experienced PE, thus we can't speculate any role for these cells. However, it would be useful to monitor Dasatinib patients who also develop side effects such as PE, colitis, autoimmune-like syndromes to observe whether there are any differences in ILC and NK cell repertoire as compared to

other TKI-treated patients or whether it could be any correlation with their clinical outcome.

It has been demonstrated that BM microenvironment in hematological malignancies played a crucial role in hematopoietic stem cell differentiation, in the acquisition of the resistance to therapy and in disease relapse [94]. TKI therapy has provided an important contribution in terms of molecular remission and prolonged survival of patients [66, 82]. However, TKI drugs did not eliminate LSC (MRD) because these cells are quiescent and resistant to TKI-induced apoptosis [109]. Moreover, TKI therapy could interfere with normal hematopoiesis and then affected the generation of ILC and NK cells. In order to understand this, we analyzed ILC and NK cells repertoire in BM of Nilotinib-treated patients at diagnosis and three months after the therapy beginning. The results show that BM-derived NK cells in Nilotinib-treated patients were enriched in $CD56^{bright}CD16^{-}$ expressing lower levels of activating receptors, any degranulation capability and lower capability to produce IFN-γ to NK cells isolated at diagnosis in the PB of the same patients. In the last years, it has been suggested that NK cells isolated from ovarian carcinoma patients may express checkpoint inhibitors such as PD-1. We evaluated the expression of PD-1 both in the PB and in the BM of our patients, at diagnosis and during therapy. However, we could never observe detectable proportions of $PD\text{-}1^{+}$NK cells (data not shown). Importantly, after three months of therapy, Nilotinib patients were in molecular remission and NK cells isolated from BM showed levels of $CD16^{+}$ cells, of degranulation capability and of IFN-γ expression comparable to those observed in NK cells isolated from patients PB.

Our preliminary data support the hypothesis that TKI may contribute to restore a functional NK cell repertoire and that Dasatinib may somehow modulate also ILC response and repertoire. It would be very important to perform parallel analyses on patients' BM and PB to obtain crucial information on the capability of immune system to control residual leukemic disease and to understand whether long lasting TKI-treated patients may achieve TFR. In the next future we hope to continue our follow up with other patients and to complete our analyses on other cell populations such as T cells, myeloid-derived suppressor cells (MDSC), regulatory T cells (T reg) and on plasma samples that we collected from patient's PB and BM.

4.5. MATERIAL AND METHODS

CML Patients- Five patients affected by CML were identified as STKI001, STKI003, STKI004, STKI005 and STKI006 and included in our study until now. We received PB samples at diagnosis and at different time intervals. STKI001, STKI004 and STKI005 received 300 mg of Nilotinib twice a day while STKI006 received 400 mg of Nilotinib twice daily. The analyses of PB were performed at different time intervals and we decided to show the data at diagnosis and after 1 month, 3 months, 6 months and 12 months (some patients were analyzed up for two years of Follow-up). All patients are still under TKI therapy. For Nilotinib-treated patients, we were able to obtain medullary blood at diagnosis and three months after starting treatment (STKI001, STKI004, STKI005, STKI006). STKI001, STKI004 and STKI005 patients were in chronic phase at diagnosis and received Nilotinib as first-line treatment. STKI006 patient was in the chronic phase when started Nilotinib treatment upon failure of a first-line treatment with Imatinib. In the present work we report data analyzed at relapse after Imatinib failure, (i.e. before starting Nilotinib) and during Nilotinib treatment. STKI003 was in the chronic phase at diagnosis and is the only patient that underwent first-line treatment with Dasatinib. All these patients achieved a complete molecular remission. The serological detection of HCMV is negative in STKI003 patient, while the STKI001 and STKI006 patients have been positive (STKI004 and STKI005 patients' serology was not received). Nilotinib-treated patients and Dasatinib-treated patient had no side effects and have not been affected by viral infections or re-activation (CMV/EBV) neither experienced any severe bacterial infections. The QRT-PCR for BCR-ABL1 mRNA transcript has been performed after the beginning of TKI therapy.

Cell isolation and plasma collection- Hematology clinic at the San Martino Polyclinic Hospital samples provided CML patients' samples. Ethical Committee approved the study and patients gave their written informed consent according to the Helsinki Declaration. Mononuclear cells were obtained by Ficoll-Lympholyte (Cedarlane, Canada) separation**.** Plasma were collected and stored at -80°.

7-color immunophenotyping- Cocktail of fluorochrome-conjugated monoclonal antibodies, human contains**:** αCD14 (clone: Tük4) FITC, αCD56 (clone: REA196) PE, αCD16 (clone: VEP13) PE αCD4 (clone: VIT4) PerCP, αCD19 (clone: LT19) PE-Vio770, αCD3 (clone: BW264/56) APC, αCD8 (clone: BW135/80) APC-Vio770, αCD45 (clone: 5B1) VioBlue (Miltenyi Biotec). Immunofluorescent staining of whole blood (lyse/no wash): 50 µL of whole blood incubate with cocktail for ten minutes in the dark at 4°C.

After, were added lysis solution and incubate for 15 minutes in the dark at room temperature. Finally, cells suspension was analyzed immediately to the flow cytometer.

Monoclonal antibodies (mAbs) and Flow Cytometry- mAbs were purchased from several companies. A full list of the mAbs utilized is provided in Table 1. All the mAbs were mouse-anti human. Cell viability were determined using Viobility 405/520 Fixable Dyes (Miltenyi Biotec) to exclude dead cells.

Intra-cytoplasmic cytokine and TF expression assays- To detect cytokines, cells are overnight stimulated with IL-12 (10 ng/ml), IL-15 (50 ng/ml), IL-18 (100 ng/ml) or IL-1β (50 ng/ml), IL-7 (50 ng/ml), IL-23 (50 ng/ml) (PeproTech, UK) in the presence of monensin (GolgiStop) or brefeldin (GolgiPlug) (BD Biosciences), respectively. For intra-cytoplasmic cytokine analyses, cells were stained for surface markers and then fixed and permeabilized with Fixation and Permeabilization Kit (BD Biosciences, New Jersey USA). Then, cells were incubated with cytokine-specific mAbs. To detect TF expression, cells were suspended in 5% BSA buffer, stained for surface markers, subsequently fixed with Transcription Factor Staining Buffer Set (eBioscience-ThermoFisher) and stained for RORγt.

CD107a degranulation assay- Patient's lymphocytes were *in vitro* stimulated for 72h with IL-15 (25ng/ml) and then incubated for were incubated in 1/1 ratio with the human erythroleukemia cell line K562 in the presence of the αCD107 for 3-hours. Monensin (GolgiStop, BD) was added after one hour of incubation to the cells. At the end of the incubation the cells were collected and marked to surface immunofluorescence and analyzed on the flow cytometer.

Real Time-Reverse Transcription-Polymerase Chain Reaction (RT-PCR) – Total RNA was extracted from mononucleated cells derived from PB and BM of CML patients at different time points (1 month, 3 months, 6 months and 9 months). RNA was synthesized into cDNA according to standard procedures in the SuperScript IV One-Step RT-PCR pre-amplification system kit (Invitrogen, Rockville, MD). The BCR-ABL1 transcripts were amplified using the following primers: 5'- GAG CAG CAG AAG AAG TGT TTC AGA-3' (BCR-P210-F exons 12/13), 5'- CAA CAG TCC TTC GAC AGC AG-3' (BCR-P190-F exon 1), 5'- CTT GGA GTG AGG CAT CTC AG-3' (ABL1-R exon 10), 5'- CAT CAT TCA ACG TGT GCC GAC GG-3' (ABL1 A fragment exon 4), 5'- GTT GCA CTC CCT CAG GTA GTC-3' (ABL1 A fragment exon 6), 5'- GAA GAA ATA CAG CCT GAC GGT G-3' (ABL1 B fragment exon 4), 5'- CGT CGG ACT TGA TGG AGA A-3' (ABL1 B fragment exon 7), 5'- TGG TAG GGG AGA ACC ACT TG-3'

(ABL1 C fragment exon 7), 5'- CCT GCA GCA AGG TAC TCA CA-3' (ABL1 C fragment exon10). Patients' samples and negative controls were analyzed in triplicate.

Table 2- List of mAbs used in the experiments.

Antigen	Antibody clone	Fluorochrome	Supplier
CD56	N901	PeCy7	Beckman-Coulter
CD159a	Z199	APC	Beckman-Coulter
CD159a	Z199	PE	Beckman-Coulter
CD158a	EB6B	APC	Beckman-Coulter
CD158a	EB6B	PE	Beckman-Coulter
CD158b1,b2	GL183	APC	Beckman-Coulter
CD158b1,b2	GL183	PE	Beckman-Coulter
CD158e1,e2	Z27.3.7	APC	Beckman-Coulter
CD158e1,e2	Z27.3.7	PE	Beckman-Coulter
CD3	SK7	APC-eFluor-780	eBioscience-ThermoFisher
CD3	SK7	eFluor450	eBioscience-ThermoFisher
CD3	BW264/56	FITC	Miltenyi Biotec
CD33	REA775	VioGreen	Miltenyi Biotec
CD33	AC104.3E3	APC	Miltenyi Biotec
CD14	TÜK4	FITC	Miltenyi Biotec
CD14	61D3	eFluor450	eBioscience-ThermoFisher
CD14	61D3	APC-eFluor-780	eBioscience-ThermoFisher
HLA-DR	AC122	PerCP	Miltenyi Biotec
CD16	REA423	FITC	Miltenyi Biotec
CD16	3G8	BV-421	BioLegend
CD16	3G8	PerCP	BioLegend
CD117	104D2	BV-421	eBioscience-ThermoFisher
CD117 (c-KIT)	104D2	PerCP-Cy5.5	BioLegend
NKG2C	FAB138P	AF-488	R&D systems
CD226 (DNAM-1)	11A8	PE	BioLegend
CD335 (NKp46)	9E2	eFluor450	BioLegend
CD336 (NKp44)	P44-8	AF-647	BioLegend
CD337 (NKp30)	P30-15	AF-647	BioLegend
CD127	A019D5	BV-421	BioLegend

Antigen	Antibody clone	Fluorochrome	Supplier
CD127	A019D5	PerCP-Cy5.5	BioLegend
CD34	4H11	PE	eBioscience-ThermoFisher
CD15	W6D3	FITC	Miltenyi Biotec
CD19	HIB19	APC-eFluor-780	eBioscience-ThermoFisher
CD19	HIB19	eFluor450	eBioscience-ThermoFisher
CD4	M-T466	VioGreen	Miltenyi Biotec
CD8	BW135/80	APC	Miltenyi Biotec
CD25	M-A251	BV-421	BD Biosciences
CD39	TU66	FITC	BD Biosciences
CD45	2D1	FITC	eBioscience-ThermoFisher
CD57	HCD57	PB	Biolegend
CD69	FN50	FITC	Miltenyi Biotec
IL-22	22URTI	PE	eBioscience-ThermoFisher
ROR-γt	AFKJS-9	PE	eBioscience-ThermoFisher
EOMES	WD1928	eFluor-660	eBioscience-ThermoFisher
IFN-γ	4S.B3	PerCP-Cy5.5	eBioscience-ThermoFisher
IFN-γ	4S.B3	eFluor450	eBioscience-ThermoFisher
TNF-α	MAb11	eFluor450	eBioscience-ThermoFisher
FOXp3	259D/C7	AF-647	BD Biosciences
CD107a	LAMP-1	PE	Miltenyi Biotec

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Effect of Tyrosin Kinase Inhibitors on NK Cell and ILC3 Development and Function

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Tyrosin kinase inhibitors (TKI) sharply improved the prognosis of Chronic Myeloid Leukemia (CML) and of Philadelphia$^+$ Acute Lymphoblastic Leukemia (Ph$^+$ALL) patients. However, TKI are not curative because of the development of resistance and lack of complete molecular remission in the majority of patients. Clinical evidences would support the notion that patient's immune system may play a key role in preventing relapses. In particular, increased proportions of terminally differentiated CD56$^+$CD16$^+$CD57$^+$ NK cells have been reported to be associated with successful Imatinib therapy discontinuation or with a deep molecular response in Dasatinib-treated patients. In view of the potential role of NK cells in immune-response against CML, it is important to study whether any TKI have an effect on the NK cell development and identify possible molecular mechanism(s) by which continuous exposure to *in vitro* TKI may influence NK cell development and repertoire. To this end, CD34$^+$ hematopoietic stem cells (HSC) were cultured in the absence or in the presence of Imatinib, Nilotinib, or Dasatinib. We show that all compounds exert an inhibitory effect on CD56$^+$ cell recovery. In addition, Dasatinib sharply skewed the repertoire of CD56$^+$ cell population, leading to an impaired recovery of CD56$^+$CD117$^-$CD16$^+$CD94/NKG2A$^+$EOMES$^+$ mature cytotoxic NK cells, while the recovery of CD56$^+$CD117$^+$CD94/NKG2A$^-$RORγt$^+$ IL-22-producing ILC3 was not affected. This effect appears to involve the Dasatinib–mediated inhibition of Src kinases and, indirectly, of STAT5-signaling activation in CD34$^+$ cells during first days of culture. Our studies, reveal a possible mechanism by which Dasatinib may interfere with the proliferation and maturation of fully competent NK cells, i.e., by targeting signaling pathways required for differentiation and survival of NK cells but not of ILC3.

Keywords: innate lymphocyte cells, NK cell development, tyrosin kinase inhibitors, CML-chronic myelogenous leukemia, ILC3

INTRODUCTION

Therapy with tyrosin kinase inhibitors (TKI) has greatly improved the prognosis of Chronic Myeloid Leukemia (CML) and of Philadelphia$^+$ Acute Lymphoblastic Leukemia (Ph$^+$ALL) patients (1–3). However, despite their efficacy, TKI cannot be considered as curative therapeutic agents, because the majority of patients develop resistance or lack complete molecular remission

(4–6). Moreover, patients undergoing life-long treatment may experience adverse effects that compromise their quality of life (6–8). Thus, during the past several years, the achievement of therapy discontinuation, allowing long treatment-free remissions (TFR) became a main area of investigation (8). Recent clinical trials indicated that ∼40% of patients with deep molecular response (DMR), who undergo therapy discontinuation, remain free of relapse for several years (8–10). Clinical evidences would support the notion that the patient's immune system may play a key role either by eradicating leukemia or by exerting a successful long-lasting control of residual leukemic cells. Indeed, patients with an efficient effector arm of their immune system display a significantly longer TFR with a DMR (11–13). Thus, ongoing major efforts are aimed to identify immune mechanisms and biomarkers that may help to select patients who are suitable for successful therapy discontinuation upon achievement of a DMR. In this context, analysis of natural killer (NK) cells, capable of a potent anti-leukemia activity, could offer a clue to identify such patients (14).

NK cells represent an important component of the innate immunity. Their function is finely tuned by inhibitory and activating receptors. Remarkably, NK cells have been shown to play an important role in the favorable clinical outcome of patients with high risk leukemias undergoing haploidentical Hematopoietic Stem Cell Transplant (15–18). NK cells can recognize and kill leukemic blasts, particularly those displaying down-regulation of HLA-class I molecules (failing to interact with HLA-specific inhibitory NK receptors such as KIR and CD94/NKG2A), and/or over-expressing ligands recognized by activating NK receptors (including Natural Cytotoxic Receptors, NCR, NKG2D and DNAM-1) (16, 19). Certain activating KIR, present only in some individuals, may also positively contribute to the anti-leukemia activity (14, 20, 21).

NK cell development occurs primarily in the bone marrow (BM), requires the expression of E4BP4 (NFIL3), Tbx21 (T-bet), eomesodermin (EOMES) transcription factors (TF), and proceeds through a multi-step process, characterized by the sequential acquisition of surface receptor markers (including CD161, CD56, CD94/NKG2A, LFA-1, CD16, KIRs, and CD57) and given functional capabilities (22–24). NK cells are developmentally related to Innate Lymphoid Cells (ILCs) as they derive from a common DNA-binding protein inhibitor (ID2)-positive hematopoietic precursor (24). Members of ILC family are thought to play a relevant role in innate defenses against pathogens, in epithelial tissue homeostasis and in lymphoid structure organization. Three main groups of ILCs have been identified: ILC1, ILC2, and ILC3, on the basis of their cytokine profile and transcription factors (TF) required for their differentiation (25). *In vitro* models of human NK cell development from umbilical cord blood (UCB)-derived $CD34^+$ cells revealed that these precursors can give rise both to NK cells and ILC3. The expression of CD94/NKG2A and LFA-1 marks $CD161^+CD56^+CD117^-CD7^+$ NK cells that express NCR, cytolytic granules and production of IFN-γ. On the other hand, the lack of expression of CD94/NKG2A and LFA-1 ($CD161^+CD56^+CD117^+CD7^-$LFA-$1^-$CD94/NKG2A$^-$) identifies a heterogeneous cell subset, that may contain both NK cell precursors and ILC3, characterized by the expression of RAR-related orphan receptor gamma (RORγt) TF and by the ability to produce IL-22 (26, 27).

In the past few years, the effects of TKI on the NK cell repertoire and function have been analyzed in several studies (28). Of note, increased proportions of terminally differentiated cytolytic $CD56^+CD16^+CD57^+$ NK cells were found in patients that achieved a successful Imatinib therapy discontinuation or in Dasatinib-treated patients with a DMR (12, 29–32). Recently, it has also been suggested that KIR genotype may represent a new biomarker for response to TKI therapy (33–35). On the other hand, previous *in vitro* studies reported conflicting results on the effect of different TKI on NK cell proliferation and function (28).

In view of the potential role of NK cells in the control of CML, it is important to study the effect of TKI not only on mature NK cells, but also on NK cells undergoing maturation. Notably, TKI may impair hematopoiesis, consequent to the inhibitory effect on c-KIT transduction pathway. Moreover, Dasatinib inhibits Src kinase, also involved in the regulation of hematopoiesis. Thus, it is possible that prolonged administration of TKI may affect NK cell differentiation from Hematopoietic Stem Cells (HSC) (24, 36–38). To explore this possibility, whether indeed TKI could influence NK cell development and repertoire, UCB-derived $CD34^+$ HSC were cultured in the absence or in the presence of Imatinib, Nilotinib, or Dasatinib. Our results show that all compounds exert an inhibitory effect on cell proliferation. In addition, Dasatinib sharply skewed the repertoire of $CD56^+$ cells, with an impaired recovery of $CD56^+CD117^-CD16^+$CD94/NKG2A$^+$EOMES$^+$ mature cytotoxic NK cells, paralleled by an enrichment of $CD56^+CD117^+$CD94/NKG2A$^-$RORγt$^+$ ILC3. This effect appears to involve the Dasatinib–mediated inhibition of Src kinases. Our studies, revealed a mechanism by which Dasatinib may interfere with the maturation of fully competent NK cells, i.e., by targeting signaling pathways required for differentiation of NK cells but not of ILC3.

MATERIALS AND METHODS

Cell Isolation and *in vitro* Culture

Liguria Cord Blood Bank provided UCB samples from healthy individuals. Ethical Committee approved the study and mothers gave their written informed consent according to the Helsinki Declaration. Mononuclear cells were obtained by Ficoll-Lympholyte (Cedarlane, Canada) separation. $CD56^-CD34^+$ cells (>98% purity) were obtained by MACS positive separation (Miltenyi Biotec, Germany). Cells were cultured in RPMI 1640 (Lonza, Belgium) containing 10% human AB serum (Biowest, France), Stem Cell Factor (SCF) (10 ng/ml), Fms-related tyrosine kinase 3 ligand (FLT3-L) (10 ng/ml), Interleukin-7 (IL-7) (20 ng/ml), Interleukin-15 (IL-15) (20 ng/ml), Interleukin-21 (IL-21) (20 ng/ml) (Miltenyi Biotec,), in the absence or in the presence of: Imatinib (IM 5 μM), Nilotinib (NIL 3, 6 μM), Dasatinib (DAS 200 nM) (Selleck Chemicals, USA) at the plasma concentration 30 min post administration, or with Dimethyl sulfoxide (DMSO) at the

corresponding concentration of the drugs (D 1:1,000/1:25,000) (Sigma-Aldrich, USA) or with KX2-391 used at different concentrations (Selleck Chemicals). We added TKI, DMSO, or KX2-391 at day 0 and at later intervals i.e., 24 h, 4, 10, or 15 days.

Monoclonal Antibodies (mAbs) and Flow Cytometry

mAbs were purchased from several companies. A full list of the mAbs utilized is provided in **Table 1**. All the mAbs were mouse-anti human, with the exception of ROR-γt mAb, Phospho-Stat3 (Tyr705)(D3A7)XP mAb, and Phospho-Stat5 (Tyr694)(D47E7) XP mAb were from Rabbit. To perform cell gating strategy we first identified morphological parameters using FSC-A vs. SSC-A. Then, we performed a further gate in which we analyze the FSC-A vs. FSC-H, in order to limit any interference due to doublets. To assess cell viability we performed analyses with Propidium Iodide and Annexin V.

Cell Cytotoxicity Assay

Cell cytotoxicity was analyzed in a 4 h 51Cr-release assay against human leukemic K562 cell line. Cells were counted, washed and plated. Effector/Target (E/T) cell ratio is 2/1. The effector target ratio was normalized to the numbers of $CD56^+$ cells present in each culturing conditions. To this end, we adjusted the number of effector cells in each condition accordingly to the $CD56^+$ cells present in the cultures and to the cell count performed simultaneously by using MACSQuant flowcytometer. Experiments were performed in duplicates. Data are expressed as percentage of target cell lysis.

Intra-cytoplasmic Cytokine, Cytolytic Granules, and TF Expression Assays

To detect cytokines, cells cultured in different conditions, were washed, suspended and over night stimulated with IL-12 (10 ng/ml), IL-15 (50 ng/ml), IL-18 (100 ng/ml), or IL-1β (50 ng/ml), IL-7 (50 ng/ml), IL-23 (50 ng/ml) (Peprotech, UK) in the presence of monensin (GolgiStop) or brefeldin (GolgiPlug) (Biosciences), respectively. For intra-cytoplasmic cytokine and cytolytic granules analyses, cells were stained for surface markers and then fixed and permeabilized with Fixation and Permeabilization Kit (BD Biosciences, New Jersey USA). Then, cells were incubated with cytokine- or Perforin-specific mAb. To detect TF expression, cells were suspended in 5% BSA buffer, stained for surface markers, subsequently fixed with Transcription Factor Staining Buffer Set (eBioscience-ThermoFisher) and stained for RORγt, and EOMES TF; instead, to detect expression of pSTAT3 and pSTAT5, cells were fixed with PFA 4% and methanol 100% and after that cells were stained with anti-pSTAT antibodies.

Statistical Analysis

Prism GraphPad software was used for statistical analysis. We considered significant $P \leq 0.05$.

TABLE 1 | List of the mAbs used in the experiments.

Antigen	Antibody clone	Fluorochrome	Supplier
CD56	N901	PeCy7	Beckman-Coulter
CD159a	Z199	APC	Beckman-Coulter
CD159a	Z199	PE	Beckman-Coulter
CD158a	EB6B	APC	Beckman-Coulter
CD158b1,b2	GL183	APC	Beckman-Coulter
CD158e1,e2	Z27.3.7	APC	Beckman-Coulter
CD158a	EB6B	PE	Beckman-Coulter
CD158b1,b2	GL183	PE	Beckman-Coulter
CD158e1,e2	Z27.3.7	PE	Beckman-Coulter
CD336 (NKp44)	Z231	PE	Beckman-Coulter
CD337 (NKp30)	Z25	PE	Beckman-Coulter
CD335 (NKp46)	BAB281	PE	Beckman-Coulter
CD33	AC104.3E3	APC	Miltenyi Biotec
CD14	TÜK4	FITC	Miltenyi Biotec
HLA-DR	AC122	PerCP	Miltenyi Biotec
CD16	REA423	FITC	Miltenyi Biotec
CD11a (LFA-1)	TS2/4	PerCP	BioLegend
CD11a (LFA-1)	TS2/4	FITC	BioLegend
CD7	CD7-6B7	FITC	BioLegend
CD335 (NKp46)	9E2	eFluor450	BioLegend
CD117 (c-KIT)	104D2	PerCP-Cy5.5	BioLegend
CD161	HP-3G10	PerCP-Cy5.5	BioLegend
CD226 (DNAM-1)	11A8	PE	BioLegend
CD94	DX22	FITC	BioLegend
CD336 (NKp44)	P44-8	AF-647	BioLegend
CD337 (NKp30)	P30-15	AF-647	BioLegend
CD16	3G8	BV-421	BioLegend
CD127	A019D5	BV-421	BioLegend
CD132	TUGh4	APC	Biolegend
CD14	61D3	eFluor450	eBioscience-ThermoFisher
CD117	104D2	BV-421	eBioscience-ThermoFisher
CD14	61D3	APC-eFluor-780	eBioscience-ThermoFisher
IL-22	22URTI	PE	eBioscience-ThermoFisher
ROR-γt	AFKJS-9	PE	eBioscience-ThermoFisher
EOMES	WD1928	eFluor-660	eBioscience-ThermoFisher
Perforin	dG9	PE	eBioscience-ThermoFisher
IFN-γ	4S.B3	eFluor450	eBioscience-ThermoFisher
TNF-α	MAb11	eFluor450	eBioscience-ThermoFisher
CXCL8	6217	PE	R & D system
pSTAT3 XP rabbit (Tyr705)	D3A7	Unconjugated	Cell signaling
pSTAT5 XP rabbit (Tyr694)	D47E7	Unconjugated	Cell signaling
Goat-anti rabbit (IgG H + L) IgG1	A27034	AF-488	Invitrogen-ThermoFisher
CD122	Mik-β2	PE	BD Pharmingen

RESULTS

TKI Inhibit *in vitro* Proliferation of Myeloid and Lymphoid Precursors From CD34$^+$ HSC

In order to analyze the effects of different TKI on *in vitro* NK cell differentiation, UCB-CD34$^+$ HSC were isolated and cultured with cytokines (SCF, FLT3-l, IL-7, IL-15 and IL-21, see section Material and Methods), suitable to promote NK cell differentiation, either in the absence (control = CTR) or in the presence of different TKI at plasmatic concentrations: Imatinib 5 μM (IM), Nilotinib 3.6 μM (NIL), Dasatinib 0.2 μM (DAS). DMSO was used as vehicle control. After 15 days of culture, cells were counted and analyzed for informative surface markers. As shown in **Figure 1A**, TKI led to a decreased mono-nucleated cell recovery that was particularly sharp in the case of Dasatinib. Evaluation of the surface staining for Annexin V after 3 and 8 days of culture suggested that the strong reduction of cell numbers detected in the presence of Dasatinib may be due to an increased programmed cell death, occurring during the first week of culture (**Figure 1B**). The analysis of informative cell surface markers indicated that the recovery of both CD33$^+$CD14$^-$ myeloid cells and CD33$^+$CD14$^+$ monocytes, was sharply reduced in the presence of all TKI analyzed (**Figure 1C**). Remarkably, all TKI induced a significant reduction of CD161$^+$CD56$^+$ absolute cell numbers as compared to controls. Also in this case, the effect was most striking in the presence of Dasatinib (**Figure 1D**). Of note, results obtained in cultures performed in the presence of Dasatinib displayed a sharp significant difference also with cultures performed in the presence of DMSO 1:25,000 (**Figures 1A,C,D**). Since cultures performed with DMSO at the final concentrations of 1:25,000 did not substantially differ in cell recovery from the CTR cultures, data on the cultures performed with DMSO at this concentration will not be shown any further.

Dasatinib Skews *in vitro* Cell Differentiation of CD161$^+$CD56$^+$ Precursors Toward ILC3

Next, we analyzed the surface phenotype of CD161$^+$CD56$^+$ cells obtained after 25 days under the culture conditions indicated above. As shown in **Figure 2A**, analysis of the receptor repertoire of CD56$^+$ cells revealed a significant reduction of CD56$^+$CD94/NKG2A$^+$ cells in the presence of Dasatinib, while the percentages of CD56$^+$CD117$^+$ cells were significantly increased. Accordingly, also the percentages of NKG2D$^+$, DNAM-1$^+$, and CD16$^+$ cells were reduced as compared to controls (**Figure 2B**). **Figure 2C** shows a representative experiment: in the presence of Dasatinib, a major decrease of percentages of CD56$^+$ cells could be detected. Moreover, the majority of CD56$^+$ cells were represented by CD117$^+$LFA-1$^-$CD94/NKG2A$^-$CD16$^-$ cells, a subset that may include both ILC3 and Stage III NK cell precursors (27). Thus, we further analyzed the expression of RORγt and Eomes TF, which allows discriminating ILC3 and stage IV/V NK cells. This analysis revealed a significant increase of CD56$^+$ RORγt$^+$ ILC3 and a significant decrease of CD56$^+$ Eomes$^+$ NK cells as compared to controls (**Figures 3A,B**), Of note, we could detect higher percentages of CD56$^+$CD117$^+$CD127$^+$(CD132$^+$) cells in cultures performed in the presence of Dasatinib as compared to controls, while CD122$^+$ cells were virtually undetectable (**Figure 3C**).

CD161$^+$CD56$^+$ Cells Developed in the Presence of Dasatinib Express Higher Percentages of IFN-γ$^+$ Cells but Display a Reduced Cytolytic Activity

The altered composition of CD56$^+$ cell subsets occurring in the presence of Dasatinib, suggests that this compound may affect NK cell differentiation and proliferation. To verify whether TKI could also affect the functional activity of CD56$^+$ cells, we analyzed the intra-cytoplasmic cytokine expression and the cytolytic activity against the NK-susceptible K562 human leukemia cell line.

CD56$^+$ lymphoid cells, developed in the absence or in the presence of different TKI, were stimulated either with IL-1β, IL-7, and IL-23, or with IL-12, IL-15, and IL-18 and analyzed for intra-cytoplasmic cytokine expression by flow cytometry. **Figures 4A,B** shows that CD56$^+$CD117$^+$CD94$^-$ ILC3 subset, generated in the presence of Dasatinib, display higher percentages of IL-22$^+$ and IL-8$^+$ cells and a slight increase of IFN-γ$^+$ cells. Interestingly, in the presence of Dasatinib, also the small CD56$^+$CD117$^{-/+}$CD94$^+$ cell subset, expressed higher proportions of IFN-γ$^+$ cells as compared to the other culturing conditions (**Figures 4A,B**). Analysis of the cytolytic activity against K562 target cells, indicated that CD56$^+$ cells generated in the presence of Nilotinib or Dasatinib, were significantly less cytolytic (**Figure 4C**). Of note, the decreased cytotoxicity, observed in the presence of Dasatinib, was associated to lower percentages of CD56$^+$ Perforin$^+$ cells as compared to controls (**Figure 4D**).

Inhibition of Src Kinases Skews CD56$^+$ Cell Repertoire Undergoing *in vitro* Cell Differentiation

It has been reported that Dasatinib, but not Imatinib and Nilotinib, exerts an inhibitory effect on the family of Src kinases: thus, it is possible that the lower numbers of CD56$^+$ cells, detected in the presence of this compound, may reflect an inhibitory effect on Src kinases occurring at the level of cell precursors (36, 39). To test this possibility, CD34$^+$HSC were cultured in appropriate cytokine mix medium in the absence or in the presence of different concentrations of KX2-391 (5, 50, 100, and 200 nM) a non-selective Src kinase inhibitor, or in the presence of Dasatinib (200 nM). After 15 days of culture, cells were counted. KX2-391 did not significantly impair cell proliferation at different drug concentrations with the exception of highest dose (200 nM), in which an extensive cell death could be detected (**Figures 5A,B**). Moreover, there were no significant variations in CD56$^+$ cell percentages in the presence of lower concentrations of KX2-391 inhibitor as compared to controls (**Figure 5C**). However, analysis of the cell surface phenotype revealed that KX2-391 could induce substantial modifications of

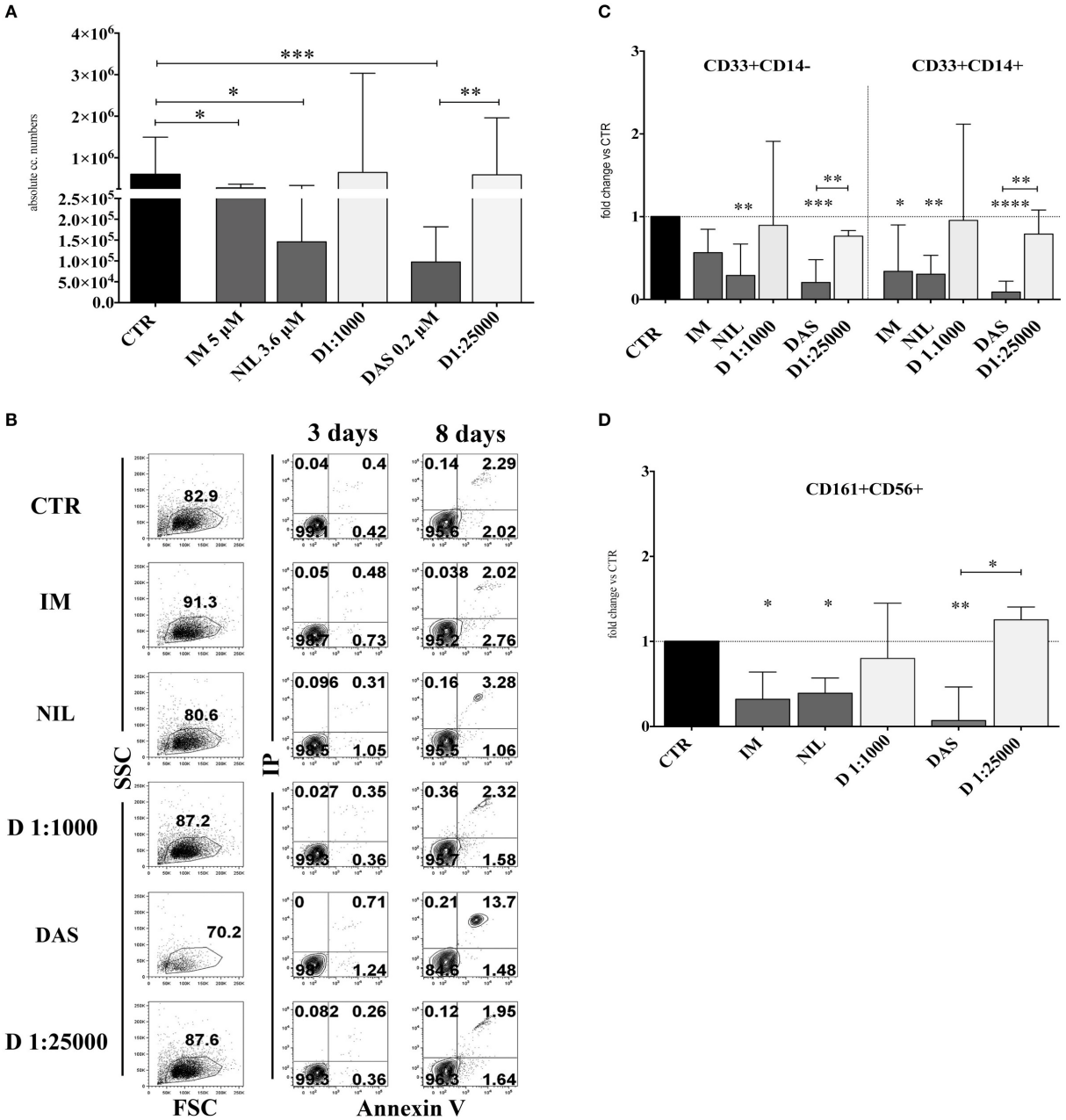

FIGURE 1 | TKI inhibit the *in vitro* cell differentiation of $CD34^+$HSC toward myeloid and lymphoid cells. UCB-$CD34^+$ cells were purified and cultured with cytokine-mix medium in the absence (CTR, control) or in the presence of different TKI: Imatinib 5 μM (IM) Nilotinib 3,6 μM (NIL), Dasatinib 0,2 μM (DAS) at the plasma peak concentrations and DMSO at the corresponding concentrations of the drugs (D 1:1000 and D 1:25000). After 15 days of culture, cells were counted and analyzed by flow-cytometry for the indicated surface markers. **(A)** The histogram shows the absolute mononucleated cell number recovery in CTR or TKI cultures. The data are represented as the median with interquartile range obtained by ten independent experiments and analyzed by Kruskal-Wallis multiple comparison test (*$p <$ 0.05;**$p < 0.005$;***$p < 0.0005$). **(B)** Dot plots show the morphological features and Annexin V/PI staining observed in the cell precursors undergoing *in vitro* NK cell differentiation in the presence of different culturing conditions: control (CTR), Imatinib 5 μM (IM), Nilotinib 3,6 μM (NIL), Dasatinib 0,2 μM (DAS) and DMSO (D 1:1000 and D 1:25000). The cells were undergone to immunofluorescence tests after 3 and 8 days of culture. **(C)** The histogram represents the fold change of absolute cell numbers of $CD33^+CD14^-$ and $CD33^+CD14^+$ cells recovered in TKI and DMSO cultures as compared to CTR, arbitrarily normalized to one. The data are expressed as the median values with interquartile range obtained by ten independent experiments. Data obtained in different culturing conditions were compared to CTR and analyzed by Wilcoxon signed-rank test (*$p < 0.05$;**$p < 0.005$;***$p <$ 0. 0005;****$p < 0.00005$). Comparison between the different TKI culturing conditions and the different DMSO diluition culturing conditions was analyzed by Kruskal–Wallis multiple comparison test (*$p < 0.05$; **$p < 0.005$). **(D)** The histogram represents the fold change of $CD161^+CD56^+$ absolute cell number recovered in the presence of TKI and DMSO as compared to CTR, arbitrarily normalized to one. Data are represented as the median values with interquartile range obtained by ten independent experiments and comparison with CTR was analyzed by Wilcoxon signed-rank test (*$p < 0.05$;**$p < 0.005$). Comparison between the different TKI culturing conditions and the different DMSO dilution culturing conditions was analyzed by Kruskal–Wallis multiple comparison test (*$p < 0.05$).

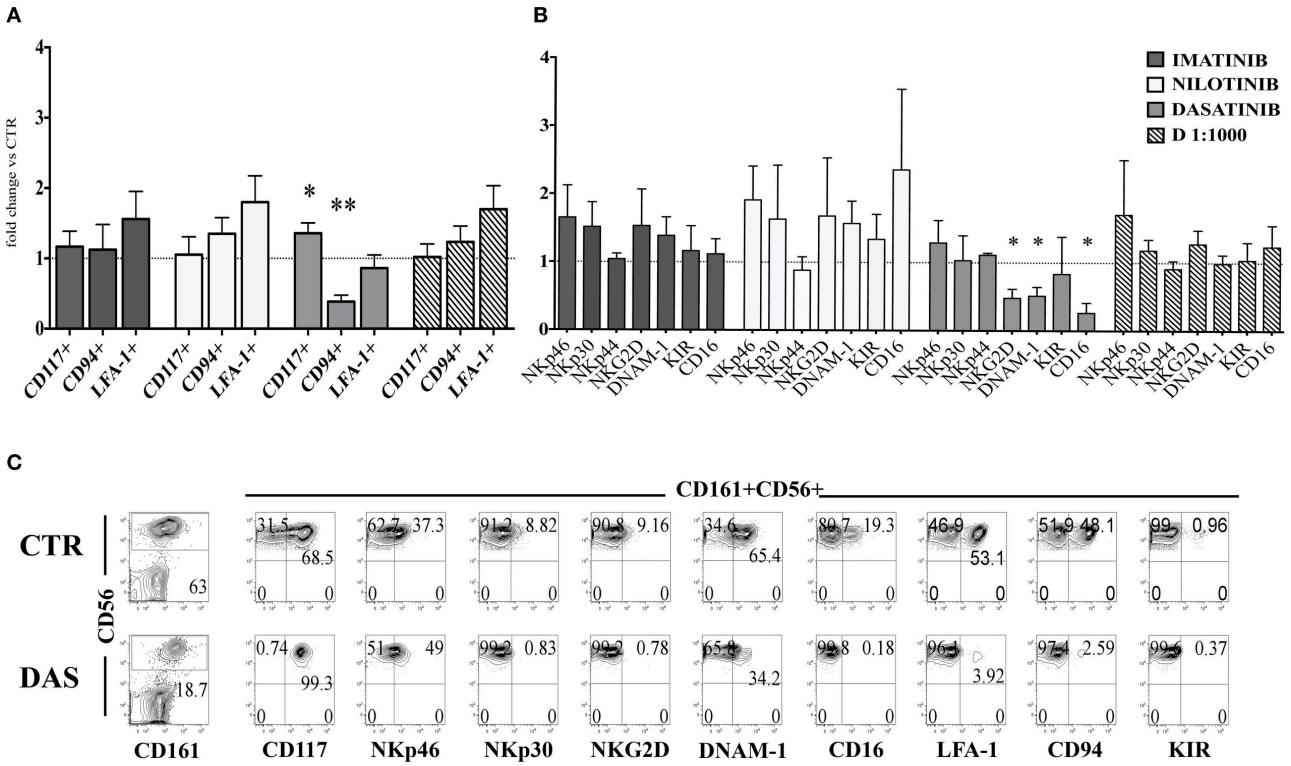

FIGURE 2 | Dasatinib inhibits NK cell generation from $CD34^+$ precursors. $CD161^+CD56^+$ cells developed in the absence (CTR) or in the presence of Imatinib 5 μM (IM), Nilotinib 3.6 μM (NIL), Dasatinib 0.2 μM (DAS) and DMSO 1:1000 (D 1:1000) were analyzed after 25 days of culture for the expression of the indicated surface markers. **(A)** The histogram represents the fold change of $CD161^+CD56^+CD117^+$, $CD161^+CD56^+CD94/NKG2A^+$, and $CD161^+CD56^+LFA\text{-}1^+$ cell percentages, detected in the presence of the indicated TKI or DMSO, as compared to CTR cultures, arbitrarily normalized to one. Data are expressed as mean values ± SEM obtained by ten independent experiments and analyzed by Wilcoxon signed-rank test (*$p < 0.05$;**$p < 0.005$). **(B)** The histogram represents the fold change of $CD161^+CD56^+$ cell percentages expressing NKp46, NKp30, NKp44, NKG2D, DNAM-1, KIR, and CD16 receptors, developed in the presence of the indicated TKI or DMSO as compared to CTR cultures, arbitrarily normalized to one. Data are expressed as mean values ± SEM of ten independent experiments and analyzed by Wilcoxon signed-rank test (*$p < 0.05$). **(C)** Flow cytometric analyses show surface staining for the indicated surface markers expressed by $CD161^+CD56^+$ cells obtained after 25 days of culture in the absence (CTR) or Dasatinib 0.2 μM (DAS). Representative experiment out of ten.

the subset composition within the $CD56^+$ cell population, similar to those detected in the presence of Dasatinib. Thus, as shown in **Figures 5D,E**, even in the presence of the lowest concentration of KX2-391 (i.e., 5 nM), there was an increased expression of RORγt TF and of CD117, paralleled by the reduced expression of Eomes TF and of CD94/NKG2A. Accordingly, $CD56^+$ cells undergoing differentiation in the presence of Dasatinib 200 nM or KX2-391 5 nM, displayed a lower cytolytic activity against K562 target cells as compared to control (**Figure 5F**).

It has been shown that Dasatinib can inhibit the Signal Transducer and Activator of Transcription (STAT) 3 and STAT 5 protein phosphorylation through the inhibition of Src kinases. Thus, the Dasatinib–mediated effects could also be due to the inhibition of STAT3/STAT5 signaling pathways. To address this issue we investigated whether Dasatinib could inhibit STAT3/STAT5 phosphorylation in $CD34^+$ cell precursors since early days of *in vitro* culture. Thus, $CD34^+$ cells were cultured with cytokine mix medium in the absence or in the presence of Dasatinib, and STATs protein phosphorylation was analyzed at different time intervals, i.e., 18/24/48 h of culture. A reduction of $pSTAT5^+$ cell percentages was detected while the percentages of $pSTAT3^+$ cells were similar or higher than those detected in control cultures at all the time intervals analyzed (**Figure 6A**). Of note, after 72 h of culture in the presence of Dasatinib, we could still observe lower $pSTAT5^+$ cell percentages as compared to control, and increase of $CD117^+$ cell percentages (**Figure 6B**). On the other hand, analysis performed at 20 days of culture, showed that $CD56^+$ cell subsets developed in the presence of Dasatinib, contained higher percentages of $pSTAT5^+$ cells as compared to controls, while myeloid cells displayed a marked reduction of both $pSTAT3^+$ and $pSTAT5^+$ cells (**Figure 6C**).

DISCUSSION

In this study we analyzed the effect of the TKI inhibitors Imatinib, Nilotinib, and Dasatinib on NK cell differentiation from UCB-derived $CD34^+$ cell precursors. We show a sharp inhibition of cell proliferation and a reduced recovery of both myeloid and $CD161^+CD56^+$ lymphoid cells. More importantly, Dasatinib skewed the subset composition of $CD161^+CD56^+$

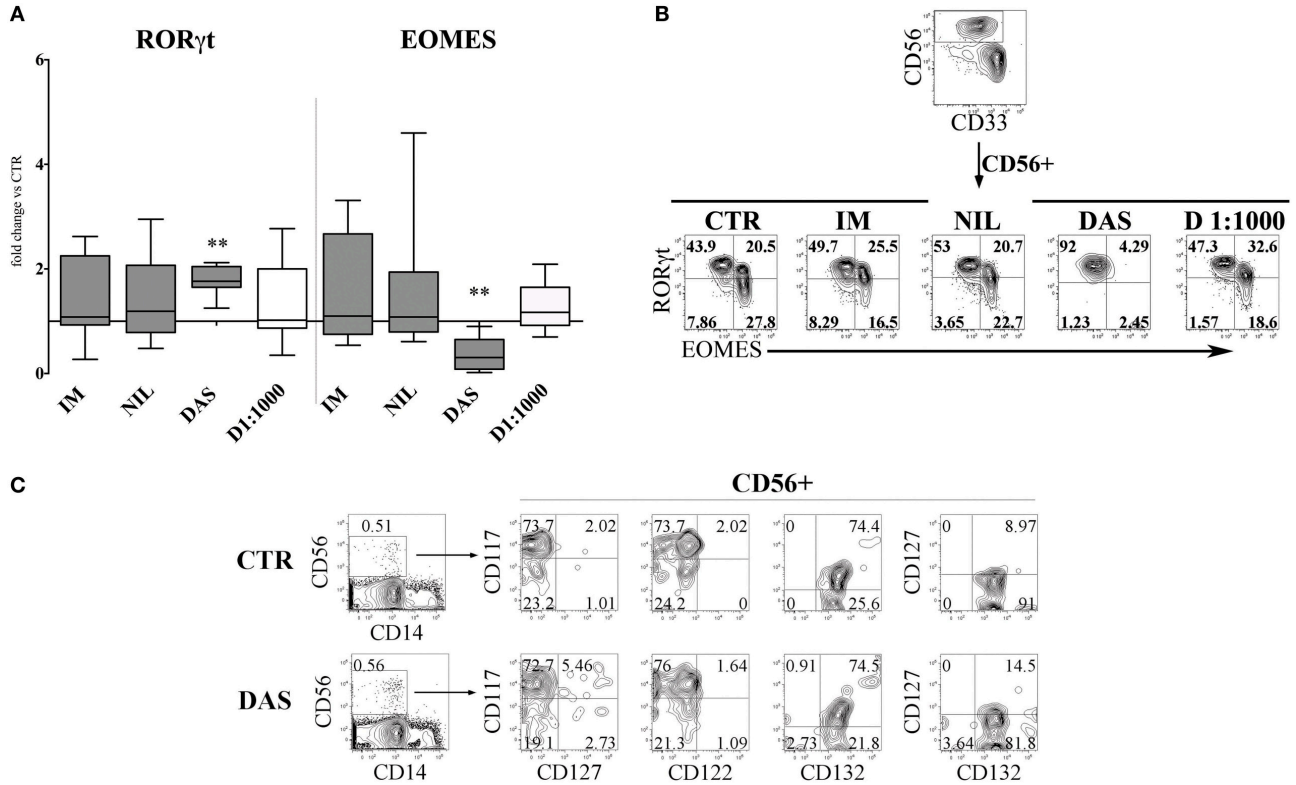

FIGURE 3 | Dasatinib affects the CD56$^+$ cell repertoire, favoring the enrichment of CD56$^+$CD117$^+$CD94/NKG2A$^-$RORγt$^+$ ILC3**.** CD161$^+$CD56$^+$ cells developed in the absence (CTR) or in the presence of Imatinib 5 μM (IM), Nilotinib 3.6 μM (NIL), Dasatinib 0.2 μM (DAS) and DMSO 1:1000 (D 1:1000) were analyzed by flow-cytometry after 25 days of culture for the expression of transcription factors (TF). **(A)** The histogram represents the fold change of CD56$^+$RORγt$^+$ and CD56$^+$EOMES$^+$ cell percentages, obtained in the presence of the indicated TKI or DMSO, as compared to CTR, arbitrarily normalized to one. Data are expressed as median values with interquartile range obtained by ten independent experiments and analyzed by Wilcoxon signed-rank test (**$p < 0.005$). **(B)** Dot plots show intra-nuclear staining of RORγt and EOMES after gating on CD33$^-$CD161$^+$CD56$^+$ cells, developed in the absence (CTR) or in the presence of the indicated TKI or DMSO. Representative experiment out of ten performed. **(C)** UCB-CD34$^+$ cells were purified and cultured with cytokine-mix medium in the absence (control=CTR) or in the presence of Dasatinib 200 nM. After 6 days of culture cells were analyzed by flow-cytometry for the indicated surface markers. Flow cytometric analysis show representative experiment out of two.

cell population favoring the generation of RORγt$^+$IL-22$^+$ ILC3 and inhibiting both proliferation and function of cytotoxic NK cells. Experiments using the KX2-391 Src-family kinase inhibitor suggest that the impairment of NK cell generation and function may be consequent to the inhibitory effect mediated by Dasatinib on Src-family kinases and the early impairment of STAT5 signaling in CD34$^+$ cell precursors.

The TKI therapy in CML or Ph$^+$ALL patients has dramatically improved the prognosis of these patients. However, the development of resistance and leukemia relapse, in particular upon therapy discontinuation, still represents a major problem. Thus, in recent years, a major research focus has been to improve both DMR and TFR (8). Notably, a positive clinical outcome in these patients appears to correlate with an efficient immune response allowing the control of the CML residual disease (13). Since NK cells are thought to play a relevant role in this process, it was important to clarify whether TKI could influence not only the repertoire and function of circulating mature NK cells, but also their development from CD34$^+$ HSC. To this end, we took advantage of an *in vitro* model of NK cell differentiation from CD34$^+$ cell precursors that allows the generation of different ILC subsets. In addition, it provides a useful tool to analyze factors/drugs that may modify such process (27, 40). In the present experiments, CD34$^+$ cells were cultured in the presence of Imatinib, Nilotinib, and Dasatinib at plasmatic peak concentrations, to reproduce drug concentrations present in PB and BM of TKI-treated patients. An inhibitory effect of TKI on precursor cell proliferation could be expected, since these compounds are known to interfere with the SCF/c-KIT (CD117) transduction pathway that plays a key role in the early steps of CD34$^+$ cell activation and proliferation (37, 41). However, the impairment of cell proliferation and recovery was associated with an increase of programmed cell death only in Dasatinib culture condition, in which apoptosis was clearly detectable during the first week of culture. Although all TKI analyzed induced a decrease of CD161$^+$CD56$^+$ cell recovery, Dasatinib exerted a more marked inhibitory effect. Moreover, only Dasatinib significantly skewed the CD161$^+$CD56$^+$ cell repertoire favoring CD117$^+$CD94$^-$LFA-1$^-$-RORγt$^+$ ILC3, while CD117$^-$CD94$^+$LFA-1$^+$Eomes$^+$ NK

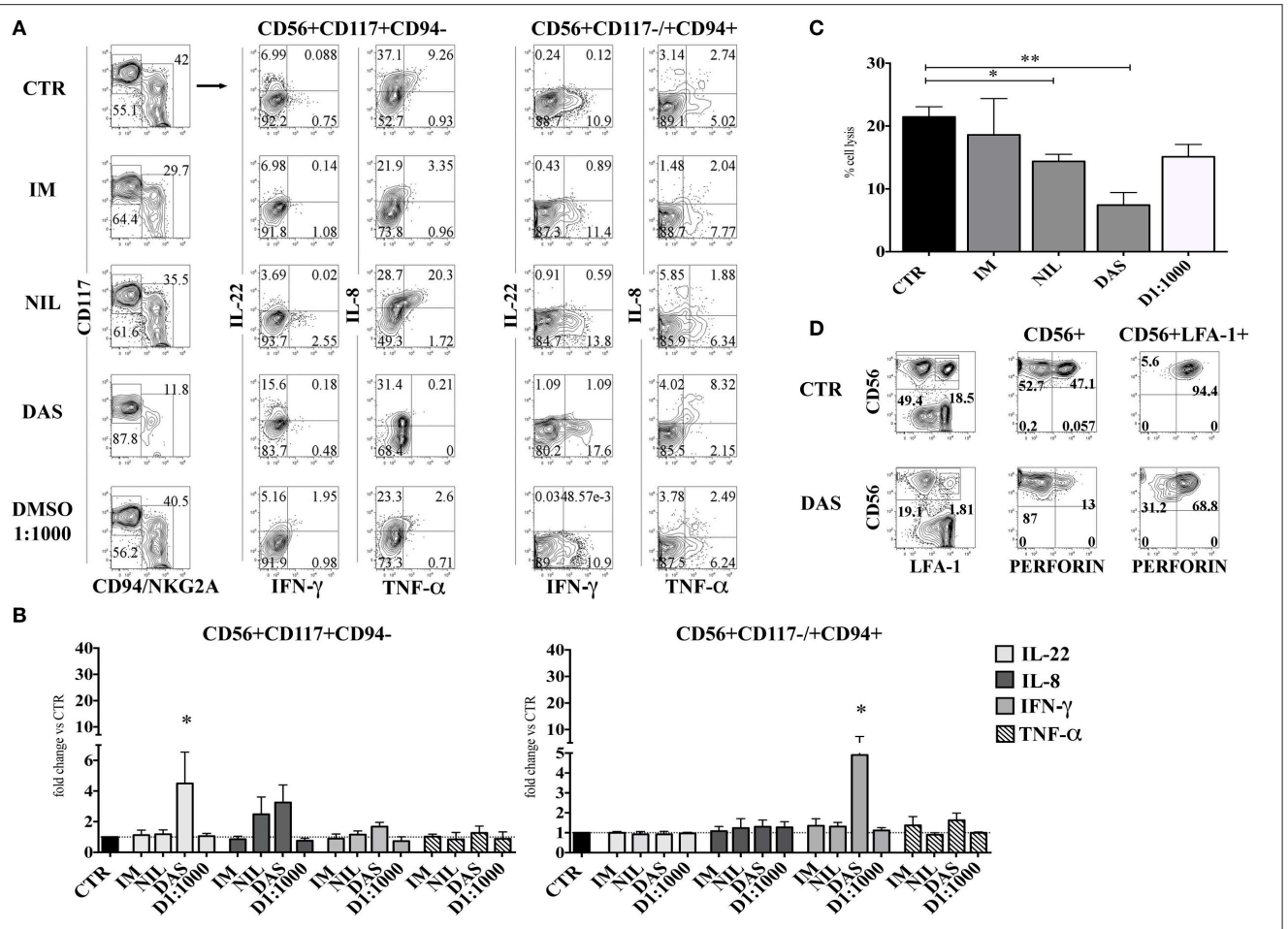

FIGURE 4 | Dasatinib increases the percentages of IL-22-producing $CD56^+CD117^+CD94^-$ and of IFN-γ-producing $CD56^+CD117^{+/-}CD94^+$ cells while inhibits cytolytic activity of $CD56^+$ cells. After 25 days $CD56^+$cells developed in the presence of Imatinib 5 μM (IM), Nilotinib 3.6 μM (NIL), Dasatinib 0.2 μM (DAS) and DMSO 1:1000 (D 1:1000) were analyzed for the intra-cytoplasmic cytokine expression and for cell cytotoxicity. **(A,B)** $CD56^+$ cells, developed in the absence or in the presence of different TKI or DMSO 1:1000 were o.n. stimulated with IL-1β+IL-7+IL-23, to induce the production of IL-22 and TNF-α and IL-8 or with IL-12+IL-15+IL-18, to favor the production of IFN-γ. Cells were analyzed for intra-cytoplasmic cytokine expression of IL-22, IL-8, IFN-γ, and TNF-α cytokines by flow cytometry. **(A)** Flow cytometry analysis show representative experiment out of seven: dot plots represent the intra-cytoplasmic cytokine staining in $CD56^+CD117^+CD94/NKG2A^-$ and $CD56^+CD117^{-/+}$ $CD94/NKG2A^+$ cell subsets. **(B)** The histograms represent the fold change of IL-22, IL-8, IFN-γ, and TNF-α positive cell percentages detected in $CD56^+CD117^+CD94/NKG2A^-$ and $CD56^+CD117^{-/+}CD94/NKG2A^+$ cell subsets upon appropriate cytokine stimulation, as compared to CTR, arbitrarily normalized to one. Data are expressed as mean percentage ± SEM of ten independent experiments and analyzed by Wilcoxon signed-rank test (*$p < 0.05$). **(C)** The histogram shows the percentage cell lysis of human leukemic line K562 by $CD161^+CD56^+$ cells. Effector/target (E/T) cell ratio was 2/1. Data are expressed as mean percentage ± SEM of four independent experiments and analyzed by Kruskal–Wallis multiple comparison test (*$p < 0.05$; **$p < 0.005$). **(D)** $CD56^+$ cells were analyzed for intra-cytoplasmic expression of Perforin. Dot plots display Perforin intra-cytoplasmic staining in total $CD56^+$ gated cells and in $CD56^+LFA\text{-}1^+(CD94^+)$ gated cells. Representative experiment out of four performed.

cells were sharply reduced. The effect of Dasatinib on ILC commitment was detectable at early stages, since analysis performed at day 6 of culture revealed an increase of $CD117^+CD127^+CD132^+$ cells, representing ILC precursors, while CD122 expression was undetectable. Previous studies on factors that may influence human ILC *in vitro* development, have shown that the presence of SCF is required to favor the *in vitro* differentiation of ILC3 in the presence of IL-7 or IL-15, while IL-15 and IL-7, alone or in combination with other pro-inflammatory cytokines, skew precursor cell differentiation toward NK cells (40, 42).

The SCF/c-kit transduction pathway involves STAT3 protein phosphorylation, IL-15 pathway preferentially uses STAT5 signaling protein, while IL-7 can exploit both (37, 43). It has been shown that Dasatinib inhibits the STAT3 and STAT5 signaling pathways through the inhibition of Src kinases, leading to a durable inhibition of STAT5 phosphorylation, but only a transient inhibition of STAT3 phosphorylation in $CD34^+$ cells isolated from CML patients at diagnosis (39). Our analysis of STAT3/STAT5 phosphorylation in $CD34^+$HSC revealed a higher and durable reduction of $pSTAT5^+$ cells as compared to $pSTAT3^+$ cells upon cell culture with Dasatinib during first

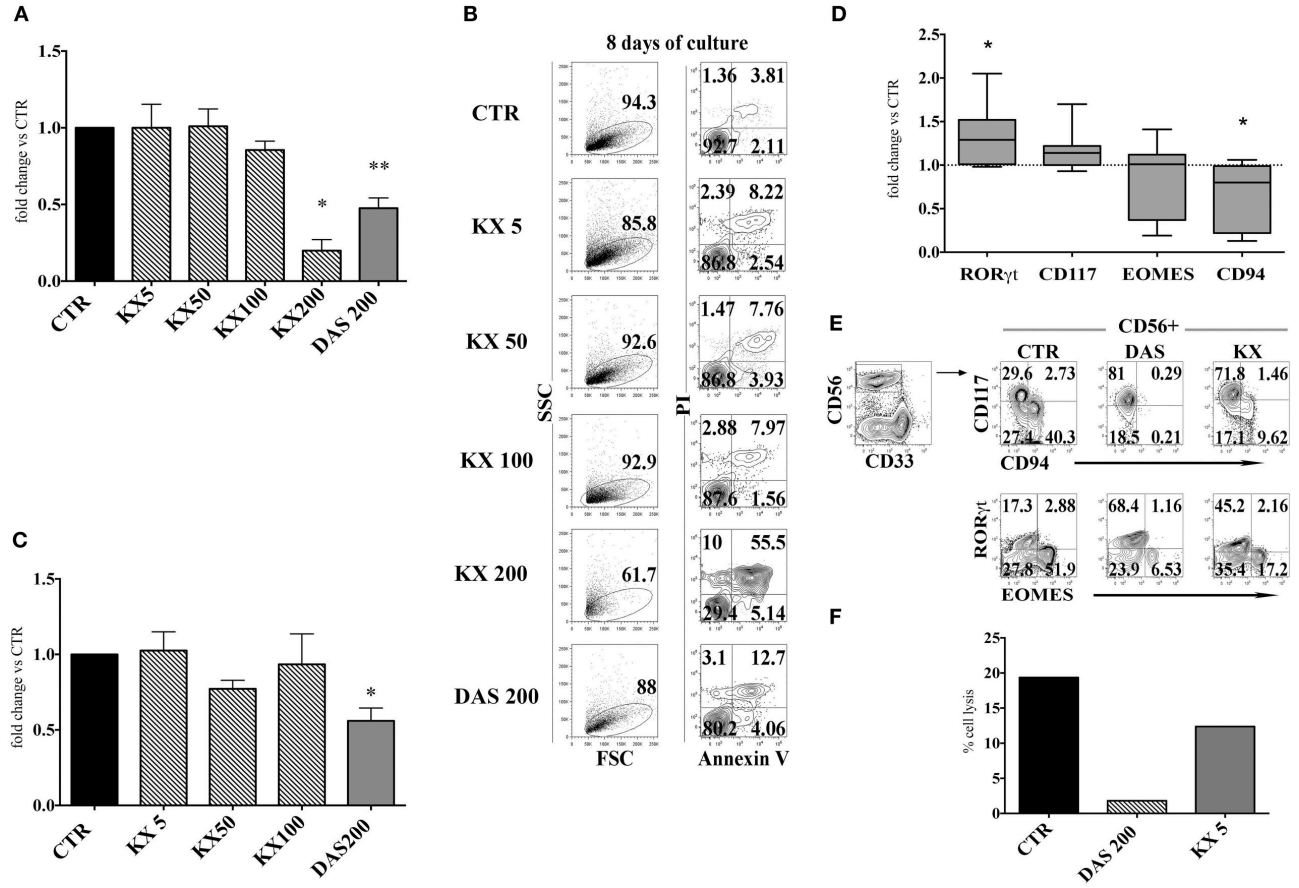

FIGURE 5 | KX2-391 Src kinases inhibitor favors the development of $CD56^+CD117^+ROR\gamma t^+$ cells, while inhibits generation and cytotoxicity of $CD56^+CD94/NKG2A^+EOMES^+$ cells. UCB-$CD34^+$ cells were purified and cultured with cytokine-mix medium in the absence (control=CTR) or in the presence of KX2-391 src kinase-inhibitor at different concentrations (5, 50, 100 nM) or Dasatinib at the plasma peak concentration (200 nM). **(A)** The histogram represents the absolute cell numbers obtained after 15 days of culture, in the presence of KX2-391 inhibitor at different concentrations and Dasatinib, as compared to control, arbitrarily normalized to one. The data are represented as mean±SEM obtained by nine independent experiments and analyzed by Wilcoxon signed-rank test (*$p <$ 0.05; **$p < 0.005$). **(B)** Dot plots show the morphological features and Annexin V/PI staining observed in the cell precursors undergoing *in vitro* NK cell differentiation in the presence of different culturing conditions: control (CTR), KX2-391 at different concentrations and Dasatinib. The cells were undergone to immunofluorescence tests after 8 days of culture. **(C)** The histogram shows the fold change of $CD161^+CD56^+$ absolute cell number recovered after 15 days of culture in the presence of KX2-391 inhibitor at different concentrations and Dasatinib, as compared to control, arbitrarily normalized to one. The data are represented as mean ±SEM obtained by nine independent experiments and analyzed by Wilcoxon signed-rank test (*$p < 0.05$). **(D)** Box and Whiskers plots represent the fold change of $CD56^+ROR\gamma t^+$, $CD56^+CD117^+$, $CD56^+EOMES^+$ and $CD56^+CD94/NKG2A^+$ cells, obtained after 25 days of culture in presence of KX2-391 5nM, as compared to control. The data are obtained by seven independent experiments and analyzed by Wilcoxon signed-rank test (*$p < 0.05$). **(E)** Dot plots show analysis of CD94/NKG2A, RORγt and EOMES TF in $CD56^+$ cells, in the absence (= CTR) or in the presence of KX2-391 5 nM or Dasatinib 200 nM. Representative experiment out of three. **(F)** The histogram represents the percentage cell lysis of human leukemic line K562 by $CD161^+CD56^+$ cells, after 27 days of culture, in the presence of KX2-391 5 nM and Dasatinib 200 nM, as compared to control. Representative experiment out of two performed.

days of culture. Thus, it is conceivable that the early expression of CD117 and CD127 on Dasatinib-treated cells may favor the SCF/CD117- and IL-7/CD127–mediated STAT3 transduction triggering pathway, thus favoring ILC precursors proliferation and survival.

Functional analyses indicate that Dasatinib does not affect cytokine production by ILC3 but rather induces increases of the percentages of $CD56^+$ $CD117^+ROR\gamma t^+$IL-22 producing cells. Interestingly, the few NK cells, undergoing differentiation in the presence of Dasatinib, contained higher percentages of IFN-γ^+cells. We also detected a slight increase of IL-8^+/IFN-γ^+ILC3 undergoing differentiation in the presence of Dasatinib. It has been reported that lymphocyte mobilization in Dasatinib-treated patients is frequently associated with adverse effects such as pleural effusion, autoimmune-like syndromes and colitis (29). Of note, ILC3 are thought to play a relevant role in intestinal inflammation (25). In addition, they may exacerbate intestinal inflammation due to their ability to differentiate toward IFN-γ-producing ILC1 cells upon IL-12 stimulation (44). Thus, the inflammatory side effects induced by Dasatinib in mucosal tissues may reflect, at least in part, increases in the production of IL-22, IL-8, and IFN-γ by ILC. Of note, the increase of IFN-γ-expressing

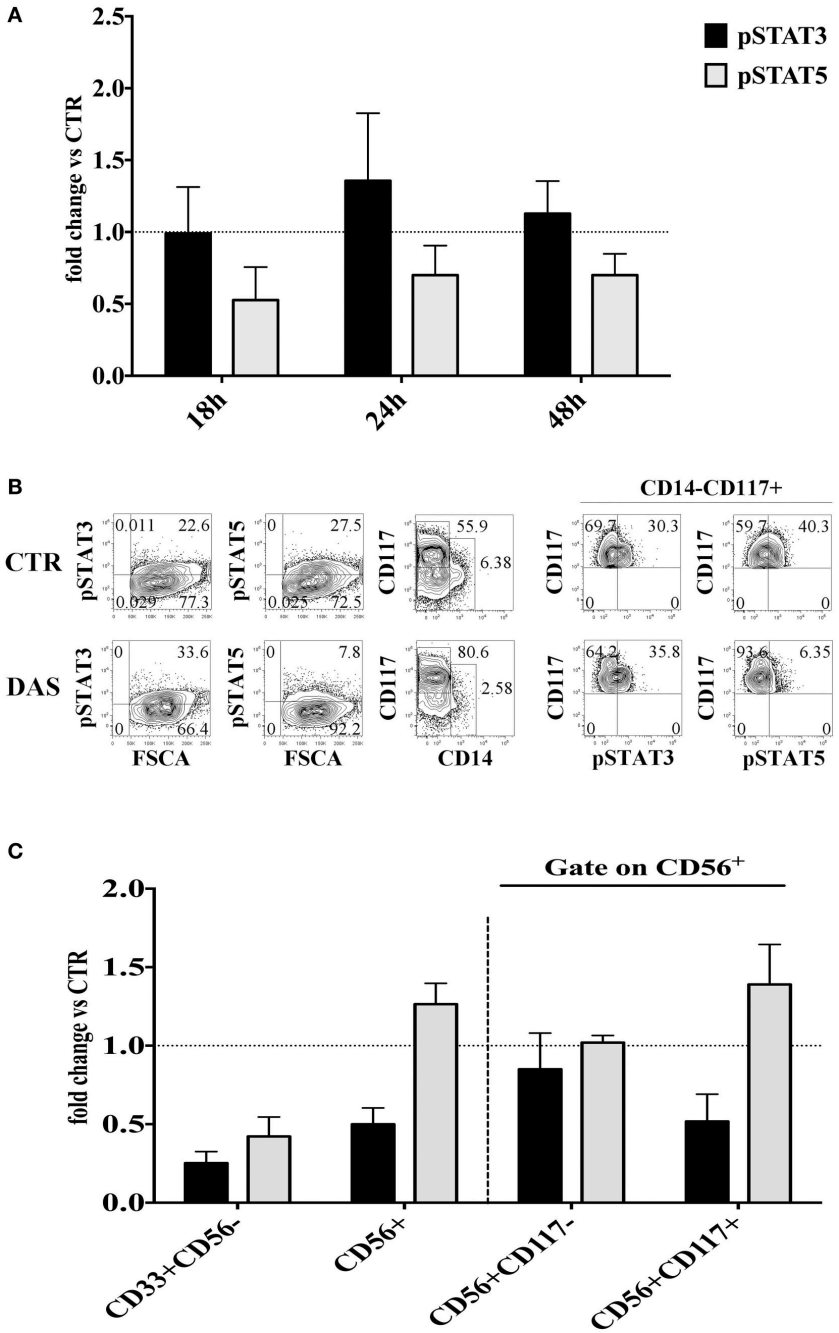

FIGURE 6 | Analysis of the Dasatinib-mediated effect on STAT3 and STAT5 phosphorylation in $CD34^+$ cells undergoing differentiation toward $CD56^+$cells at different culture time intervals. **(A)** UCB-$CD34^+$ cells were isolated and cultured with cytokine-mix medium, in the absence or in the presence of Dasatinib 200 nM for 18, 24, and 48 h. Cells were analyzed for the expression of pSTAT3 and pSTAT5 signaling proteins. The histograms show the fold changes of $CD34^+pSTAT3^+$ and $CD34^+pSTAT5^+$ positive cell percentages detected in the presence of Dasatinib, as compared to control, arbitrarily normalized to one. Data are expressed as mean values ± SEM obtained by four independent experiments. **(B)** UCB-$CD34^+$ cells were isolated and cultured with cytokine-mix medium, in the absence or in the presence of Dasatinib 200 nM for 72 h. Flow cytometry analyses show the intra-cytoplasmic expression of pSTAT3 and pSTAT5 on total cell population and on $CD14^-CD117^+$ gated cells. **(C)** $CD56^+$ cells obtained from UCB-$CD34^+$ cells after 20 days of culture, in the absence (CTR) or in the presence of Dasatinib 200 nM were analyzed for the expression of pSTAT3 and pSTAT5 signaling proteins. The histogram shows the fold change of $CD56^+pSTAT3^+$ and $CD56^+pSTAT5^+$ cell percentages detected in the presence of Dasatinib, as compared to control, arbitrarily normalized to one. Data are expressed as mean values ± SEM obtained by four independent experiments.

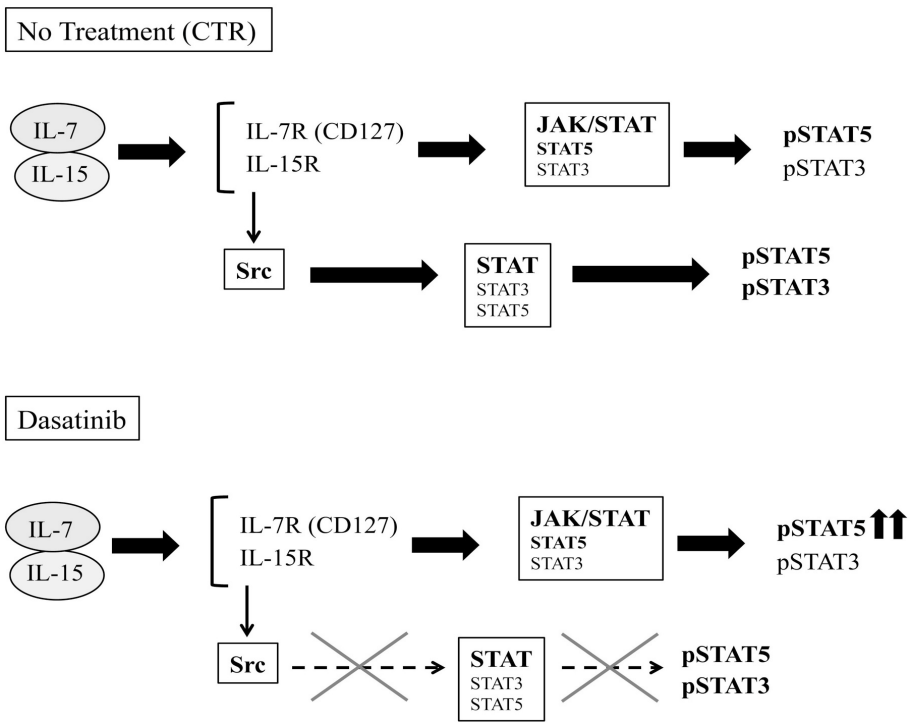

FIGURE 7 | Schematic representation of potential effects of Dasatinib on IL-7/IL-15 transduction pathways. IL-7 and IL-15 cytokines can signal both through the JAK-STAT and the Src pathways.After 20 days of culture, in the control condition, the cytokine receptors engagement by IL-7 and IL-15 leads both to JAK- and Src-STAT-mediated recruitment and phosphorylation. In the presence of Dasatinib, Src-mediated STAT phosphorylation pathway is abrogated, while cytokine-driven JAK-activation pathway would provide compensatory signals, allowing STAT5 phosphorylation.

$CD56^+$ cells detectable after 25 days of culture in the presence of Dasatinib, paralleled the increase of $CD56^+pSTAT5^+$cell percentages. These data may suggest that a prolonged exposure to IL-7 and IL-15 may provide compensatory signals, allowing lymphoid cell cytokine production even in the presence of a chronic exposure to Dasatinib (45) (**Figure 7**).

The cytolytic activity of $CD56^+$ NK cells developed in the presence of Dasatinib was impaired. It is conceivable that the reduction of cytotoxicity may reflect the lower number of mature NK cells however, we could also detect a decrease of Perforin content in the few mature NK cells present in Dasatinib cultures. In addition, it should be considered the inhibitory effect exerted by Dasatinib on Src kinases and on ERK protein phosphorylation (p-ERK), required for the cytolytic degranulation process (39, 46, 47). Indeed, our experiments using KX2-391 inhibitor would confirm that the effect on the $CD56^+$ cell repertoire induced by Dasatinib, may be due to the impairment of Src kinase pathway. Notably, KX2-391 induced massive cell apoptosis at the concentration of 200 nM while at lower concentrations the proportions of Annexin V^+ cells were low. However, the use of KX2-391 at lower concentrations skewed the $CD56^+$ cell repertoire toward $CD56^+CD117^+CD94/NKG2A^-$ $ROR\gamma t^+$ ILC3, similar to what detected in cultures performed in the presence of Dasatinib.

Studies in mice models have suggested that IL-15/STAT5 pathway may represent a central node in NK cell homeostasis and in the TF network that instructs ILC development (48–51). Our data indicate that exposure to Src kinase inhibitors may play a relevant a role in the inhibition of human NK cell development and in the acquisition of cytolytic activity, while the cytokine-driven STAT3 and STAT5 activation pathway may overcome Dasatinib-mediated inhibition and favor the preferential survival of $CD117^+$ $CD127^+$ ILC3 precursors and the subsequent cytokine production by both mature ILC3 and NK cells detectable at later time culture intervals.

It has been reported that Dasatinib, may induce cytotoxic $CD56^+CD57^+$ NK cell mobilization in the PB and BM of TKI-treated patients (29–31, 52). It is conceivable that different effects detectable *in vitro* vs. *in vivo* of Dasatinib-treatment may be consequent to the short half-life of the drug in plasma vs. its stable levels in cell cultures (53). Notably, it has been suggested that the incidence of lymphocytosis does not correlate with DMR, while the numbers and percentages of circulating cytotoxic NK cells and CTL were significantly higher in patients with DMR as compared to those with no DMR (30). The high degree of heterogeneity of cases analyzed may contribute to the variability of responses: patients may undergo different TKI administration protocols, and the immune system status (immunosuppression, viral reactivation, therapy-related BM/lymphoid organs exhaustion) may greatly influence immune cell repertoire and function, having an impact on the clinical outcome of CML-treated patients (13).

In conclusion, our results suggest that Dasatinib may affect NK cell development, and offer new clues to better understand the signaling pathways that regulate development and proliferation of precursors toward NK cells and other ILC. The Dasatinib-induced skewing of ILC differentiation toward ILC3 and increasing of IFN-γ producing cells should be considered in patients with severe therapy-induced side effects or with limited responses to therapy. Moreover, in Dasatinib-treated patients, it will be important to better characterize both the lymphoid cell precursors and the mature cytolytic effector cells present in patients BM, i.e., the site where drug-resistant leukemic clones primarily reside (54). Finally, our study, could offer a clue for identifying new tools to design individualized timing and dosing of Dasatinib administration in order to obtain optimal responses without compromising the NK cell-based immunotherapeutic intervention.

ETHICS STATEMENT

The ethics committe that approved the study was Comitato Etico Regionale Liguria- Ospedale San Martino. Samples were from Liguria Cord Blood Bank. This study was carried out in accordance with the recommendations of UE guidelines for Good Clinical Practice and Comitato Etico Regionale Liguria-Ospedale San Martino with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Comitato Etico Regionale Liguria- Ospedale San Martino.

AUTHOR CONTRIBUTIONS

LD designed the experimental plan and performed experiments. EM designed the first set of experiments. LM analyzed results. CV designed the experimental plan, analyzed results and wrote the paper. MCM analyzed results. All the authors contributed to the critical review of the manuscript. CV and MM share senior authorship.

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